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Allergen-specific IgE levels and the ability of IgE-allergen complexes to cross-link determine the extent of CD23-mediated T-cell activation

Sergio Villazala-Merino, MSc[a], **Azahara Rodriguez-Dominguez, MSc**[b], **Victoria Stanek, MSc**[a], **Nicholas J. Campion, MD**[a], **Pia Gattinger, PhD**[b], **Gerhard Hofer, MSc**[c], **Renate Froeschl, MSc**[d], **Ingrid Fae, MSc**[e], **Christian Lupinek, MD**[b], **Susanne Vrtala, PhD**[b], **Heimo Breiteneder, PhD**[f], **Walter Keller, PhD**[c], **Thomas Perkmann, MD**[d], **Ryosuke Nakamura, PhD**[g], **Winfried F. Pickl, MD**[h], **Rudolf Valenta, MD**[b,i,j], **Julia Eckl-Dorna, MD, PhD**[a], **Verena Niederberger, MD**[a]

[a]Department of Otorhinolaryngology, Medical University of Vienna, Vienna, Austria

[b]Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

[c]Institute of Molecular Biosciences, BioTechMed Graz, University of Graz, Graz, Austria

[d]Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria

[e]Department for Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Vienna, Austria

[f]Division of Medical Biotechnology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

[g]Division of Medicinal Safety Science, National Institute of Health Sciences, Kanagawa, Japan

[h]Institute of Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

[i]Laboratory of Immunopathology, Department of Clinical Immunology and Allergy, Sechenov First Moscow State Medical University, Moscow, Russia

[j]NRC Institute of Immunology FMBA of Russia, Moscow, Russia

Abstract

Background—CD23 mediates IgE-facilitated allergen presentation and subsequent allergen-specific T-cell activation in allergic patients.

Corresponding author: Julia Eckl-Dorna, MD, PhD, Department of Otorhinolaryngology, Research laboratories 8H, Medical University of Vienna, General Hospital of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria. julia.eckl-dorna@meduniwien.ac.at.

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Objective—We sought to investigate key factors regulating IgE-facilitated allergen presentation through CD23 and subsequent T-cell activation.

Methods—To study T-cell activation by free allergens and different types of IgE–Bet v 1 complexes, we used a molecular model based on monoclonal human Bet v 1–specific IgE, monomeric and oligomeric Bet v 1 allergen, an MHC-matched CD23-expressing B-cell line, and a T-cell line expressing a human Bet v 1–specific T-cell receptor. The ability to cross-link Fcε receptors of complexes consisting of either IgE and monomeric Bet v 1 or IgE and oligomeric Bet v 1 was studied in human FcεRI-expressing basophils. T-cell proliferation by monomeric or oligomeric Bet v 1, which cross-links Fcε receptors to a different extent, was studied in allergic patients' PBMCs with and without CD23-expressing B cells.

Results—In our model non–cross-linking IgE–Bet v 1 monomer complexes, as well as cross-linking IgE–Bet v 1 oligomer complexes, induced T-cell activation, which was dependent on the concentration of specific IgE. However, T-cell activation by cross-linking IgE–Bet v 1 oligomer complexes was approximately 125-fold more efficient. Relevant T-cell proliferation occurred in allergic patients' PBMCs only in the presence of B cells, and its magnitude depended on the ability of IgE–Bet v 1 complexes to cross-link CD23.

Conclusion—The extent of CD23-mediated T-cell activation depends on the concentration of allergen-specific IgE and the cross-linking ability of IgE-allergen complexes.

Keywords

Allergen; CD23; Bet v 1; facilitated allergen presentation; IgE; birch pollen allergy; cross-linking; T-cell activation; IgE-allergen complex

CD23, the low-affinity receptor for IgE, is mainly expressed on B cells of allergic patients, where it binds allergen-specific IgE and thus plays an important role in IgE-facilitated allergen presentation (IgE-FAP) to T cells and subsequent T-cell activation.[1–3] CD23 seems to be also expressed on gut and respiratory epithelial cells mediating allergen uptake and transcytosis.[4–6]

However, it seems that a major function of CD23 is to mediate IgE-FAP.[1,7,8] In fact, it has been shown by *in vitro* experiments that IgE-FAP can activate specific T cells at much lower allergen concentrations compared with IgE-independent allergen presentation.[7,9] Presentation of allergen-IgE complexes through CD23 induces potent activation of T cells accompanied by the release of proinflammatory T_H2 cytokines already at very low allergen concentrations and thus might play an important role in T cell–mediated allergic inflammation *in vivo*.[7] Therefore CD23-IgE–mediated allergen presentation is a potential target for the treatment of allergic inflammation.[1] In this context it has been shown that allergen-specific IgG antibodies block IgE-allergen recognition, inhibit proliferation of allergen-specific $CD4^+$ T cells, and release of proinflammatory T_H2 cytokines, such as IL-4 and IL-5.[10–15] The importance of CD23 in IgE-FAP and T-cell activation has further been demonstrated by the fact that therapeutic anti-CD23 antibodies, such as Lumiliximab, lead to a reduction of allergen-induced T-cell activation.[16]

By using molecular model systems, it has been demonstrated that CD23-expressing B cells and recombinant forms of CD23 bind monomeric IgE, as well as complexes consisting of allergen and IgE.[17,18] Furthermore, it has been shown that the CD23 surface density on B cells of allergic patients is associated with IgE levels and determines IgE-facilitated allergen uptake and, consequently, activation of allergen-specific T cells.[3] Interestingly, it was found that the IgE repertoire complexity seems to influence the process of IgE-FAP.[19] However, it has not yet been studied in detail whether levels of allergen-specific IgE have an influence on the magnitude of IgE-FAP and T-cell activation. Likewise, it has not yet been studied in detail whether there is a difference in the intensity of T-cell activation depending on whether IgE-FAP occurs through monomeric IgE-allergen complexes without cross-linking of CD23 or through CD23–cross-linking allergen-IgE complexes.

To answer these questions, we have established a molecular and cellular model based on a purified human IgE mAb specific for the major birch pollen allergen Bet v 1, defined monomeric and oligomeric forms of Bet v 1, MHC-matched CD23-expressing B cells, and a T-cell line expressing a human Bet v 1–specific T-cell receptor (TCR). The possible clinical relevance of the results obtained in the molecular and cellular model was studied in cultured cells obtained from patients with birch pollen allergy. Our study reveals that levels of allergen-specific IgE and the ability of IgE-allergen complexes to cross-link CD23 dictate the extent of CD23-mediated allergen presentation and subsequent T-cell activation.

Methods

Bet v 1 monomer, Bet v 1 oligomer, and chimeric Bip 1 IgE

rBet v 1 monomer was produced in *Escherichia coli* and purified by using acidic/salt precipitation and subsequent ion-exchange chromatography, as previously described.[20] Three copies of the Bet v 1 sequence were linked in the plasmid pET-17b to engineer the rBet v 1 oligomer, which was expressed in *E coli* and purified.[21]

Chimeric Bip 1 IgE (CB1 IgE) is an IgE mAb[22] composed of a human IgE heavy chain and the variable region and the light chain from a mouse anti–Bet v 1 IgG_1 antibody.[23] CB1 IgE was purified by means of affinity chromatography using the anti-IgE antibody mAb 12[22] and stored in PBS frozen at –20°C until use.

Cell culture

Human EBV-transformed B cells expressing CD23[3] were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, Mass) supplemented with 10% FBS (Thermo Fisher Scientific), 5 mmol/L HEPES (Thermo Fisher Scientific), 0.05 nmol/L β-mercaptoethanol (Thermo Fisher Scientific), 20 U/mL penicillin, and 20 μg/mL streptomycin (Thermo Fisher Scientific) at 37°C in a 5% CO_2 atmosphere.

Jurkat T cells, which had been engineered to express a TCR specific for Bet v 1 (peptides 142-153) and a luciferase reporter gene under the control of the IL-2 promotor,[24] were cultured in Iscove modified Dulbecco medium (IMDM; Thermo Fisher Scientific) supplemented with 10% FBS, 20 U/mL penicillin, and 20 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere.

Rat basophilic leukemia (RBL) cells (RS-ALT8) expressing the human high-affinity IgE receptor[25] were maintained in Eagle minimum essential medium supplemented with 10% FBS, 2 mmol/L L-glutamine (Thermo Fisher Scientific), 100 U/mL penicillin, 100 μg/mL streptomycin, 200 μg/mL Geneticin (Thermo Fisher Scientific), and 200 μg/mL Hygromycin B (Thermo Fisher Scientific) at 37°C in a 5% CO_2 atmosphere.

PBMCs from patients allergic to birch pollen were cultured in Ultraculture medium (Lonza, Basel, Switzerland) supplemented with 50 μg/mL gentamicin (Thermo Fisher Scientific) and 1× Glutamax (Thermo Fisher Scientific) at 37°C in a 5% CO_2 atmosphere.

Allergen presentation assay

Aliquots (100 μL) of 5×10^4 human EBV-transformed B cells per well were seeded in 96-well V-bottom plates. IgE–Bet v 1 complexes were prepared by mixing different concentrations of CB1 IgE and recombinant monomeric or oligomeric Bet v 1 (ie, complexes composed of 26 nmol/L CB1 IgE and 294 nmol/L Bet v 1 monomer or oligomer further diluted in 5-fold steps) in complete RPMI 1640 medium. Alternatively, CB1 IgE (starting at 26 nmol/L) was diluted in 5-fold steps and complexed with 59 nmol/L Bet v 1 monomer or oligomer. Furthermore, experiments were also performed only with allergen or CB1 IgE alone. Complexes or reactants were preincubated for 1 hour at 37°C, added to EBV- transformed B cells, and cultivated at 37°C in a 5% CO_2 atmosphere for 3 hours. Then plates were centrifuged at 1500 rpm at room temperature for 5 minutes, and supernatants were discarded. Next, aliquots of 200 μL containing 1×10^5 Bet v 1–specific Jurkat T cells were added per well and cultivated in IMDM medium with EBV-transformed B cells at 37°C in a 5% CO_2 atmosphere for 6 hours. Jurkat T cells without EBV-transformed B cells and stimulated with 12.5 μg/mL PHA (Sigma-Aldrich, Saint Louis, Mo) and 100 nmol/L phorbol 12-myristate 13-acetate (Sigma-Aldrich) served as positive controls, and cells cultured in medium alone were used as negative controls. After incubation, cells were lysed with freshly prepared luciferase cell-culture lysis reagent (Promega, Madison, Wis) by incubating them on a shaker at room temperature for 10 minutes. Lysates were frozen at −20°C until luciferase release was measured in a Luminoskan Ascent Luminometer (Thermo Fisher Scientific).[26] Background signal corresponding to release of luciferase from cells stimulated with medium alone was used to normalize results from the different measurements. All these experiments were performed in triplicates for each experimental condition, and results represent means ± SDs.

EBV-transformed B cells were incubated with either 30 μg/mL anti-human CD23 blocking antibody (clone M-L233; BD Biosciences, San Jose, Calif) or matching isotype (clone MOPC-21; BD Biosciences) before the addition of reactants, IgE–Bet v 1 complexes, or medium alone to study the influence of CD23 on allergen presentation. All experiments were performed in triplicates. Data represent means ± SDs.

EBV-transformed B cells were incubated with either 20 μg/mL anti-human pan-HLA blocking antibody (clone Tu36; BD Biosciences) or matching isotype (clone G155-178; BD Biosciences) before the addition of reactants, IgE–Bet v 1 complexes, or medium alone and then during the whole experiment to evaluate the influence of MHC class II on allergen presentation. All experiments were performed in triplicates. Data represent means ± SDs.

One hundred seventy-five thousand EBV-transformed B cells were primed with IgE–Bet v 1 complexes, reactants, or medium alone in a 24-well plate (700 μL/well RPMI medium) and then coincubated with 350,000 Jurkat T cells (700 μL/well IMDM medium) in a 24-well plate in the presence or absence of separation by a 0.4 μm polyester Transwell (Corning, Corning, NY; Jurkat T cells in the lower compartment and primed EBV-B cells in the upper compartment) to analyze the extent of the involvement of cell-to-cell contact in mediating CD23 IgE allergen presentation. All experiments were performed in duplicates. Data represent means ± SDs.

RBL activation assay

RBL cells were plated in complete Eagle minimum essential medium, as described in the cellculture section, in 96-well sterile tissue culture plates (50 μL/well) with 0.05 μg/mL CB1 IgE or with allergic patients' sera (1:10 dilution) in Tyrode buffer (ie, Tyrode salts dissolved in water according to the manufacturer (Sigma-Aldrich), 0.02M $NaHCO_3$, 1% w/v BSA and 50% v/v D2O, pH 7.2). Cells were incubated overnight at 37°C in a 5% CO_2 atmosphere. The next day, the cells were washed 3 times with washing buffer (Tyrode buffer [Tyrode salts; Sigma-Aldrich] in 0.02 mol/L $NaHCO_3$, pH 7.2) and 1% BSA [Sigma-Aldrich] in H_2O). Then 8 different concentrations of Bet v 1 monomer and Bet v 1 oligomer (starting concentration at 294 nmol/L in 5-fold-step serial dilutions) were added to the cells.

In another set of experiments, 10 different equimolar amounts of Bet v 1 monomer or Bet v 1 oligomer (starting concentration, 23.53 nmol/L in 4-fold-step serial dilution) were added to the cells loaded with the individual patients' sera. After the addition of allergen, cells were incubated at 37°C for 1 hour. For control purposes, cells were loaded with serum from a patient with birch pollen allergy overnight and then incubated with Bet v 1 (10 ng/mL). Also, cells loaded overnight with 1 μg/mL CB1 IgE were incubated with 1 μg/mL goat anti-human IgE (positive controls; SeraCare, Milford, Mass). Cells without serum or antibody but only allergen in different concentrations were used as negative controls. All allergen and serum samples were diluted in Tyrode buffer. Release of β-hexosaminidase into the medium from activated RBL cells was determined by using a fluorometric assay with 4-methylumbelliferyl-N-acetyl-b-D-glucosaminide as a substrate (0.1 mmol/L in 100 mmol/L citrate, pH 4.5). The reaction was stopped with 0.25 mol/L glycine buffer after incubation at room temperature for 60 minutes. The plate was read on an Infinite M200 PRO series plate reader (TECAN Austria GmbH, Grodig, Austria) using 380-nm excitation and 440-nm emission filters. β-Hexosaminidase release was calculated, as previously described.[27,28] All measurements were performed in triplicates and represent means ± SDs.

Characterization of allergic patients and quantification of allergen-specific IgE

Patients with birch pollen allergy were characterized according to demographic and clinical data, as previously described (Table I).[29] Total serum and Bet v 1–specific IgE levels were measured by ImmunoCAP (Thermo Fisher Scientific).

HLA-DR genotyping

Ethylenediamine tetra-acetic acid blood samples (9 mL) were obtained from patients with birch pollen allergy with approval of the Ethics Committee of the Medical University of

Vienna (EK508/2011) after written informed consent had been obtained. HLA-DR alleles were typed by using next-generation sequencing, as previously described.[30]

Preparation of PBMCs with and without B cells

Heparinized blood samples (60 mL) were obtained from patients with birch pollen allergy with approval of the Ethics Committee of the Medical University of Vienna (EK508/2011) after written informed consent had been obtained. PBMCs were isolated by using Ficoll (GE Healthcare, Uppsala, Sweden) density gradient centrifugation and washed with PBS. For depletion of B cells, isolated PBMCs were washed twice with 2 mmol/L ethylenediamine tetra-acetic acid (pH 7.2; Sigma-Aldrich) and 0.5% FBS in PBS (separation buffer). Then PBMCs were resuspended in the same buffer (80 μL of separation buffer per 10^7 cells) and incubated with CD19 microbeads (20 μL of CD19 microbeads per 10^7 cells; Miltenyi Biotech, Bergisch Gladbach, Germany). Cells and beads were incubated for 15 minutes at 4°C, washed again with separation buffer before they were resuspended in 500 μL of separation buffer, and applied to an LD column (Miltenyi Biotech) to remove B cells. B cell–depleted PBMCs were obtained by washing the LD column twice with 1 mL of separation buffer. The purity of the B cell–depleted PBMC preparations (>95%) was confirmed by using flow cytometry with anti-human CD19–allophycocyanin (APC; clone HIB 19; Thermo Fisher Scientific). Untouched PBMCs were subjected to the same washing, incubation steps and filtration through LD columns as B cell–depleted PBMCs without the addition of CD19 microbeads to obtain the PBMC preparation.

Allergen-induced T-cell proliferation

PBMCs or B cell–depleted PBMCs were resuspended in 5% FBS in PBS at a concentration of 1×10^7 cells/mL and CT Violet (CellTrace Violet cell proliferation kit; Thermo Fisher Scientific) was added to a final concentration of 5 μmol/L. Cells were incubated at room temperature under light protection for 5 minutes and washed 3 times with 10 volumes of 5% FBS in PBS. Then cells were resuspended in Ultraculture medium, plated in aliquots of 3×10^5 PBMCs (100 μL/well) in a 96-well U-bottom plate, and 5 different equimolar concentrations of monomeric or oligomeric Bet v 1 were added (starting concentration of 1.15 μmol/L and 2-fold dilutions). After 7 days, cells were stained with eFluor780 viability dye (Thermo Fisher Scientific) in PBS at 4°C for 30 minutes, washed in 0.1% BSA in PBS (fluorescence-activated cell sorting [FACS] buffer), and blocked with 10% mouse serum (BD Bioscience) in FACS buffer at 4°C for 20 minutes. Then cells were washed with FACS buffer and stained with anti-human CD3-APC (clone OKT3; Thermo Fisher Scientific) for 20 minutes at 4°C under light protection. Samples were analyzed in a FACSCanto II cytometer (BD Biosciences). At least 2×10^4 events were acquired per sample and analyzed with FlowJo Software (version 10; FlowJo, Ashland, Ore). T cells were identified based on CD3 expression of T cells, which had proliferated, by low staining for CT Violet. The percentage of T cells that had proliferated was normalized to the percentage of T cells, which had proliferated in unstimulated conditions. All experiments were performed in triplicates. Data represent means ± SDs of the median of triplicate results obtained for each of the 5 patients.

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Negative staining electron microscopy and marker expression

Please see the Methods section in this article's Online Repository at www.jacionline.org.

Statistics

The unpaired Student *t* test with the Welch correction was used to compare 2 single conditions. Two-way ANOVA was used to compare paired conditions in different experimental groups, as indicated in the respective figure legends (GraphPad Prism 7; GraphPad Software, La Jolla, Calif). A *P* value of .05 or less was considered significant.

Results

Allergen-specific model to study different types of allergen presentation regarding T-cell activation

To establish a model for allergen-specific T-cell activation to compare free allergen and different types of allergen-IgE complexes, we selected defined reagents. The recombinant major birch pollen allergen Bet v 1, termed the Bet v 1 monomer, is a strictly monomeric protein, whereas the rBet v 1 trimer, termed the Bet v 1 oligomer, is expressed and purified as a large oligomeric protein aggregate.[21,31] Recombinant monoclonal Bet v 1–specific IgE containing a human IgE constant region and variable domains of the mouse Bet v 1–specific IgG_1 mAb Bip 1,[22] termed CB1 IgE,[3,17] was used for IgE-FAP. For allergen presentation, EBV-transformed B cells expressing high levels of CD23 (see Fig E1 in this article's Online Repository at www.jacionline.org) were used, and Bet v 1–specific Jurkat T cells expressing a TCR specific for the Bet v 1 peptide spanning amino acids 142 to 153 and a luciferase reporter gene under the control of the IL-2 promotor served as a read-out system for induction of allergen-specific T-cell activation.[24]

Importantly, it is established that the Bet v 1 monomer in combination with CB1 IgE does not cross-link IgE receptors, whereas the Bet v 1 oligomer in combination with IgE cross-links IgE receptors.[32] Thus it is possible to form small IgE-allergen complexes consisting of CB1 IgE and Bet v 1 monomer that do not cross-link CD23 and large IgE-allergen complexes consisting of CB1 IgE and Bet v 1 oligomer that do cross-link CD23 (see Fig E2 in this article's Online Repository at www.jacionline.org).

IgE–Bet v 1 oligomer complexes induce stronger T-cell activation through CD23 than IgE–Bet v 1 monomer complexes

First, we compared specific T-cell activation induced by CD23-expressing human EBV-transformed B cells bearing complexes consisting of monomeric or oligomeric Bet v 1 (12 nmol/L) bound to CB1 IgE (1.05 nmol/L). The concentration of Bet v 1 oligomer was kept equimolar to the concentration of the Bet v 1 monomer (12 nmol/L) to ensure the presence of an identical number of allergen molecules in the differently composed complexes. Specific T-cell activation was significantly greater when cells were stimulated with IgE–Bet v 1 oligomer complexes than with IgE–Bet v 1 monomer complexes ($P < .05$; Fig 1, *A*). Neither Bet v 1 alone in any of its forms (monomeric or oligomeric) nor IgE alone triggered relevant specific T-cell activation of greater than the background signal (Fig 1, *A*).

To investigate whether the observed effect of allergen-IgE complexes on T cells was induced through CD23, we added a blocking anti-CD23 antibody to EBV-transformed B cells before addition of the complexes. Blocking of CD23 led to complete abrogation of the specific T-cell activation induced by CB1 IgE–Bet v 1 monomer and CB1–IgE–Bet v 1 oligomer complexes ($P < .001$). The addition of an isotype control did not affect activation of specific T cells through CD23-mediated FAP (Fig 1, *B*).

IgE-FAP through CD23 is dependent on presentation through MHC class II and cell-to-cell contact

We studied the role of MHC class II in IgE-FAP by blocking MHC class II on the surfaces of EBV-transformed B cells with an anti–pan-HLA antibody. As a result of MHC class II blocking, CB1 IgE–Bet v 1 monomer or oligomer complexes did not induce any specific T-cell activation of greater than the background level when compared with matching isotype or untreated samples ($P < .001$; Fig 1, *C*).

We were also interested in evaluating the role of cell-to-cell contact in specific T-cell activation by CD23-mediated FAP. To address this question, we cocultured EBV-transformed B cells primed with reactants and separated by a Transwell membrane from Jurkat T cells. Primed B-cell/T-cell cocultures without transwells were used as controls (untreated). We observed no specific T-cell activation when EBV-transformed B cells and Jurkat T cells were cocultured separated by a transwell compared with controls without transwells, where CB1 IgE–Bet v 1 complexes induced specific T-cell activation ($P < .001$; Fig 1, *D*).

IgE–Bet v 1 oligomer complexes induce T-cell activation at approximately 125-fold lower concentrations than IgE–Bet v 1 monomer complexes

Next, we investigated the dose response of T-cell activation induced by IgE–Bet v 1 oligomer and IgE–Bet v 1 monomer complexes. CB1 IgE–Bet v 1 monomer or CB1 IgE–Bet v 1 oligomer complexes were prepared by mixing rBet v 1 at 294 nmol/L (equimolar for both rBet v 1 forms) and 26 nmol/L CB1 IgE to ensure excess of allergen over IgE. The resulting complexes were diluted 8 times in 5-fold steps, thereby keeping the IgE/Bet v 1 ratio constant at each dilution. CB1 IgE–Bet v 1 oligomer complexes induced significantly greater rates of specific T-cell activation, resulting in a range of allergen concentrations between 58.8 and 0.47 nmol/L. Complexes containing the monomer induced relevant T-cell activation at greater than baseline levels until the third dilution (12 nmol/L Bet v 1), whereas approximately 125 times lower concentrations of IgE–Bet v 1 oligomer complexes were needed for activation (0.09 nmol/L Bet v 1; Fig 2, *A*). Likewise, an approximately 125-fold lower concentration of IgE–Bet v 1 oligomer complexes was still sufficient to induce T-cell activation at a level comparable with that obtained with IgE–Bet v 1 monomer complexes when comparing each of the different dilutions (Fig 2, *A*). There was no upregulation of costimulatory molecules (HLA-DR, CD86, and CD80) on EBV-transformed B cells observed in any of the conditions tested (see Fig E3 in this article's Online Repository at www.jacionline.org). Specific T-cell activation on non–IgE-mediated presentation by B cells with free Bet v 1 monomer or oligomer occurred only at a very high allergen concentration (294 nmol/L), although that difference was not statistically significant. CB1 IgE without

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allergen did not have any effect on T-cell activation at any of the concentrations analyzed in our experiments (Fig 2, *B*).

CD23-mediated allergen-specific T-cell activation increases with greater IgE concentration

To investigate the influence of IgE concentrations on CD23-mediated allergen presentation and subsequent T-cell activation, we prepared IgE–Bet v 1 complexes using 8 different concentrations of CB1 IgE, starting with a concentration of 26 nmol/L and diluting it in 5-fold steps, and complexed those with a constant concentration of rBet v 1 monomer or oligomer of 59 nmol/L. Under the given experimental conditions, we had an initial CB1 IgE/Bet v 1 ratio of 1:2, which progressively decreased, leading to an excess of Bet v 1 in the preparations. Results obtained with CB1 IgE–Bet v 1 monomer showed that the extent of T-cell activation depends on the concentration of IgE (Fig 3). The greatest level of T-cell activation was obtained with 26 nmol/L CB1 IgE. T-cell activation decreased when IgE was diluted, although the allergen concentration was kept constant, and at each of the consecutive dilution steps of IgE, allergen was in excess to IgE (Fig 3). This result is interesting because we could show that the IgE–Bet v 1 monomer complexes did not cross-link IgE receptors, as demonstrated in a basophil activation experiment that showed that IgE–Bet v 1 monomer complexes do not activate basophils (Fig 4). Therefore the extent of T-cell activation obtained with non–cross-linking IgE-allergen complexes depends on the concentration of IgE in the complex, with high IgE levels yielding the greatest level of T-cell activation.

Similar results were obtained with IgE–Bet v 1 oligomer complexes (Fig 3), although, as observed in Fig 2, *A*, IgE–Bet v 1 oligomer complexes were again approximately 125-fold more effective in inducing specific T-cell activation when comparing the different dilutions. Also, for IgE–Bet v 1 oligomer complexes, the greatest T-cell proliferations were observed with high CB1 IgE concentrations, and a reduction of the CB1 IgE concentration led to a decrease in T-cell activation (Fig 3). As already observed earlier by Sellge et al,[32] for mast cell activation, we found that IgE–Bet v 1 oligomer complexes induced dose-dependent activation of basophils and thus represent IgE-allergen complexes that can cross-link IgE receptors (Fig 4).

Fig 4 shows the direct comparison of IgE–Bet v 1 monomer complexes and IgE–Bet v 1 oligomer complexes regarding basophil activation. CB1 IgE–Bet v 1 monomer complexes did not induce any release of β-hexosaminidase at greater than the background level at any concentration and therefore did not cross-link FcεRI. By contrast, CB1 IgE–Bet v 1 oligomer complexes cross-linked FcεRI and induced dose-dependent mediator release at the 2 greatest concentrations (294 and 59 nmol/L, $P < .001$, Fig 4).

In patients with birch pollen allergy, Bet v 1–specific T-cell proliferation depends on B cells and the ability of allergen to cross-link receptor-bound IgE

Fig 5 shows proliferation of T cells collected from patients with birch pollen allergy assessed by using CT Violet dilution and gating on $CD3^+$ cells on addition of different equimolar concentrations of Bet v 1 monomer and Bet v 1 oligomer in the presence (Fig 5, *A*) and absence (Fig 5, *B*) of B cells. We tested PBMCs from 5 patients with birch pollen

allergy whose HLA-DR genotypes were different and whose Bet v 1–specific IgE levels were high and accounted for a high percentage of their total IgE levels (Table I). On incubation with Bet v 1 monomer and oligomer for 48 and 72 hours, B cells upregulated the expression of HLA-DR and CD86 in a comparable manner, whereas CD80 expression was not increased (see Fig E4 in this article's Online Repository at www.jacionline.org).

T-cell proliferation induced by Bet v 1 monomer increased with increasing antigen concentration and was greater than T-cell proliferation induced by Bet v 1 oligomer, reaching a significant difference for the concentration of 0.575 μmol/L ($P < .01$; Fig 5, *A*). After depletion of B cells from PBMCs, no relevant T-cell proliferation was observed with Bet v 1 monomer or Bet v 1 oligomer over the complete range of concentrations tested (Fig 5, *B*).

These results were initially surprising because in the strictly molecular model based on monoclonal Bet v 1–specific CB1 IgE, Bet v 1 oligomer induced much stronger T-cell activation than Bet v 1 monomer. However, for patients' polyclonal IgE, it has been shown that Bet v 1 monomer is a much stronger cross-linker of receptor-bound IgE than Bet v 1 oligomer.[21,31] Therefore we compared the ability of Bet v 1 monomer and Bet v 1 oligomer in RBL assays to cross-link the polyclonal IgE obtained from the 5 patients studied in the T-cell proliferation experiments (Fig 6). Results obtained showed that the Bet v 1 monomer is a much stronger cross-linker of patients' polyclonal IgE bound to FcεRI on basophils than Bet v 1 oligomer (Fig 6). Basophil activation was significantly stronger with Bet v 1 monomer for each of the 5 patients investigated (Fig 6). These experiments, although not directly related to T-cell activation, confirm that for polyclonal IgE from patients with birch pollen allergy, Bet v 1 monomer is a stronger cross-linker than Bet v 1 oligomer.

Discussion

Several *in vitro* studies indicate that allergen-specific activation of T cells in allergic patients is much more efficient when allergen is presented through IgE bound to CD23 on B cells (ie, by IgE-FAP) compared with allergen presentation without the involvement of IgE.[7,9,10] Key findings of these experiments were that much less allergen is needed to activate T cells when presented through IgE-FAP and that the process is dependent on CD23 because it can be blocked with anti-CD23 antibodies.[7,9,10] In the course of respiratory allergy, only very small amounts of allergen enter the body and can come into contact with cells of the adaptive immune system. Allergen uptake through respiratory exposure is low, and only a small percentage of inhaled allergens becomes systemic because of the barrier function of the respiratory mucosa. According to calculations that can be made based on the allergen contents of grass pollen (ie, 1 grass pollen grain contains approximately 0.1 ng of Phl p 5 allergen)[33] and the ability of allergens to penetrate respiratory epithelial cell layers (ie, less than a few percent),[34] it might be expected that less than 1 μg of allergen per day enters the body during a day of high grass pollen exposure. Therefore it is conceivable that IgE-FAP is a major mechanism for the activation of allergen-specific T cells during allergen exposure and plays an important role in T cell–mediated allergic inflammation in allergic patients.

Our study has elucidated 2 important and fundamental factors that control the efficacy of T-cell activation by CD23-mediated IgE-FAP. We found that IgE-FAP, which induces cross-linking of CD23, is at least 100-fold more efficient in inducing T-cell activation compared with IgE-FAP without cross-linking of CD23 (Fig 7, lower part). When performing our experiments, we have also confirmed that both forms of IgE-FAP (with monoclonal or polyclonal CD23-bound IgE) are much more efficient in activating allergen-specific T cells compared with allergen presentation without the involvement of IgE. The enhancement of allergen presentation and subsequent T-cell activation by non–cross-linking IgE-allergen complexes might be explained by spontaneous and ligand-induced endocytosis of CD23, which was described previously.[35] IgE-FAP without cross-linking of CD23 might play an important role for the presentation of IgE-reactive allergen-derived haptens, which can develop through the activity of endogenous proteases in allergen sources, such as in pollen and house dust mites.[36–39] Such IgE-reactive haptens do not elicit immediate-type symptoms because they do not trigger mast cell or basophil activation[40–42] but might induce T cell–mediated inflammation when they contain T-cell epitopes. However, our study demonstrates that T-cell activation is strongest when IgE-FAP is induced through cross-linking of CD23, a mechanism similar to allergen-induced cross-linking of IgE bound to the high-affinity receptor for IgE (FcεRI) on mast cells and basophils. In fact, we have analyzed our IgE-allergen complexes regarding their potency to cross-link receptor-bound IgE in parallel using basophil activation to verify their cross-linking abilities in a biological assay (Fig 4 and Fig 7, upper part).[32]

The second important finding of our study is that the efficacy of T-cell activation increases with increasing concentrations of allergen-specific IgE bound to CD23 (Fig 3). In fact, we know that allergen exposure induces increases in systemic allergen-specific IgE levels and induces greater sensitivity of mast cells and basophils in allergic patients.[43] Our findings indicate that increases in allergen-specific IgE levels might also increase IgE-FAP and, consequently, allergen-specific T-cell activation. This would be in agreement with a recent study demonstrating that CD23 surface density on B cells of allergic patients is associated with IgE levels and determines IgE-FAP and T-cell activation.[3]

Our initial results were obtained in defined cellular systems (ie, using a CD23-expressing B-cell line and a T-cell line expressing an allergen-specific TCR) and by using defined molecules (ie, monoclonal allergen-specific IgE and allergen derivatives with and without cross-linking ability). In this model we demonstrate also that CD23-mediated FAP requires MHC class II and cell-to-cell contact because they can be blocked by anti–pan-HLA antibodies and by separating antigen-presenting cells and T cells with transwell membranes, respectively.

Therefore we also performed experiments with primary cells from allergic patients to confirm the possible clinical relevance of our results. For this purpose, we obtained PBMCs from patients with birch pollen allergy and stimulated them with Bet v 1 forms with high and low ability to cross-link receptor-bound IgE, as demonstrated by parallel basophil activation experiments (Fig 6). These experiments confirmed that strong T-cell activation was obtained with IgE-allergen complexes with high cross-linking ability. Furthermore, we demonstrated that in the peripheral blood of allergic patients, B cells are the crucial allergen-

presenting cells because depletion of B cells reduced allergen-specific T-cell activation (Fig 5).

Thus our results demonstrate that IgE-FAP by CD23-bearing B cells is a major mechanism for activation of allergen-specific T cells, at least in the blood of allergic patients, and depends on the concentration of allergen-specific IgE and the ability of IgE-allergen complexes to cross-link IgE receptors. These results might explain why therapeutic strategies targeting the interaction of allergen and IgE, such as allergen-specific immunotherapy inducing allergen-specific IgG blocking antibodies[44] or passive immunization with allergen-specific IgG blocking antibodies,[45] also ameliorate T cell–mediated allergic inflammation and might be a basis for new therapeutic strategies targeting T cell–mediated allergic inflammation.

Methods

CD23 expression on EBV B cells

EBV-transformed B cells were stained for viability with eFluor780 viability dye (Thermo Fisher Scientific) in PBS for 30 minutes at 4°C. Subsequently, cells were washed with FACS buffer and blocked in 10% mouse serum (BD Biosciences) in FACS buffer for 20 minutes at 4°C. Cells were washed with FACS buffer and stained with anti-human CD23-phycoerythrin (clone EBVCS2; Thermo Fisher Scientific) for 20 minutes at 4°C under light protection. Samples were analyzed in a FACSCanto II cytometer (BD Biosciences). At least 20,000 events were acquired per sample and analyzed with FlowJo Software (version 10; FlowJo).

Negative staining electron microscopy

CB1 IgE and Bet v 1 monomer or oligomer were complexed in borate-buffered saline (10 mmol/L sodium borate and 150 mmol/L NaCl [pH 8.2]) at 4°C overnight (CB1 IgE, 4.7 μg/mL; Bet v 1 monomer and oligomer, 4.7 μg/mL). Samples were floated onto a 23.5-nm carbon layer, stained with 2% uranyl formate (Polysciences Europe, Eppelheim, Germany), and transferred to a T 600-Cu grid (Ted Pella, Redding, Calif). Samples were then viewed under an electron microscope (EM-900 TEM; Carl Zeiss, Oberkochen, Germany).

HLA-DR, CD80, and CD86 expression

EBV-transformed B cells were primed with 3 different concentrations of CB1 IgE–Bet v 1 monomer or oligomer complexes (ie, complexes composed of 26 nmol/L CB1 IgE and 294 nmol/L Bet v 1 monomer or oligomer further diluted in 5-fold steps) in complete RPMI 1640 medium. Furthermore, EBV-transformed B cells were also stimulated with allergen or CB1 IgE alone. Complexes or reactants were preincubated for 1 hour at 37°C, added to EBV-transformed B cells, and cultivated at 37°C in a 5% CO_2 atmosphere for 3 hours. Then plates were centrifuged at 1500 rpm at room temperature for 5 minutes, and supernatants were discarded. Next, EBV-transformed B cells were cocultured either together with 1×10^5 Bet v 1–specific Jurkat T cells per well or alone in 200 μL/well IMDM at 37°C in a 5% CO_2 atmosphere for 6 hours. Then, EBV-transfiormed B cells were stained with 1:2000 dilution eFluor 450 viability dye (Thermo Fisher Scientific) in PBS at 4°C for 20 minutes, washed in 0.1% BSA in PBS (FACS buffer), and blocked with 10% mouse serum (BD Biosciences) in

FACS buffer at 4°C for 20 minutes. Then cells were washed with FACS buffer and stained with 1:50 anti-human CD19-APC (clone HIB19; Thermo Fisher Scientific), 1:50 anti-human HLA-DR–fluorescein isothiocyanate (clone L243; Thermo Fisher Scientific), 1:25 anti-human CD86-phycoerythrin (clone 2331; BD Biosciences), and 1:10 anti-human CD80-phycoerythrin/Cy7 (clone 2D10; BioLegend, San Diego, Calif) for 20 minutes at 4°C under light protection. Samples were analyzed in a FACS-Canto II cytometer (BD Biosciences). At least 2×10^4 events were acquired per sample and analyzed with FlowJo software (version 10; FlowJo). HLA-DR, CD86, and CD80 mean fluorescence intensity (MFI) values were normalized to the MFI values of respective unstimulated samples. Data represent means ± SDs of duplicate results.

Heparinized blood samples (20 mL) were obtained from 5 patients with birch pollen allergy with approval of the Ethics Committee of the Medical University of Vienna (EK508/2011) after written informed consent had been obtained. PBMCs were isolated by using Ficoll (GE Healthcare, Uppsala, Sweden) density gradient centrifugation and washed with PBS. Three hundred thousand PBMCs were plated per well in a 96-well V-bottom plate in serum free Ultraculture medium (100 μL/well; Lonza, Basel, Switzerland), and 2 different concentrations of Bet v 1 monomer or oligomer (1.15 and 0.575 μmol/L) were added. Cells were incubated for 48 or 72 hours at 37°C, respectively. After 48 and 72 hours of culture, cells were analyzed by using flow cytometry, as described in the paragraph above. B cells were identified based on their expression of CD19, and expression of HLA-DR, CD86, and CD80 in $CD19^+$ cells was then analyzed. The MFIs of $HLA\text{-}DR^+$ and $CD80^+$ B cells were normalized to the values of the unstimulated samples. The percentage of $CD86^+$ B cells was also normalized to the percentage of $CD86^+$ B cells in unstimulated samples. All experiments were performed in duplicates. Data points represent means ± SDs of the average of duplicate results obtained for each of the 5 patients.

Extended Data

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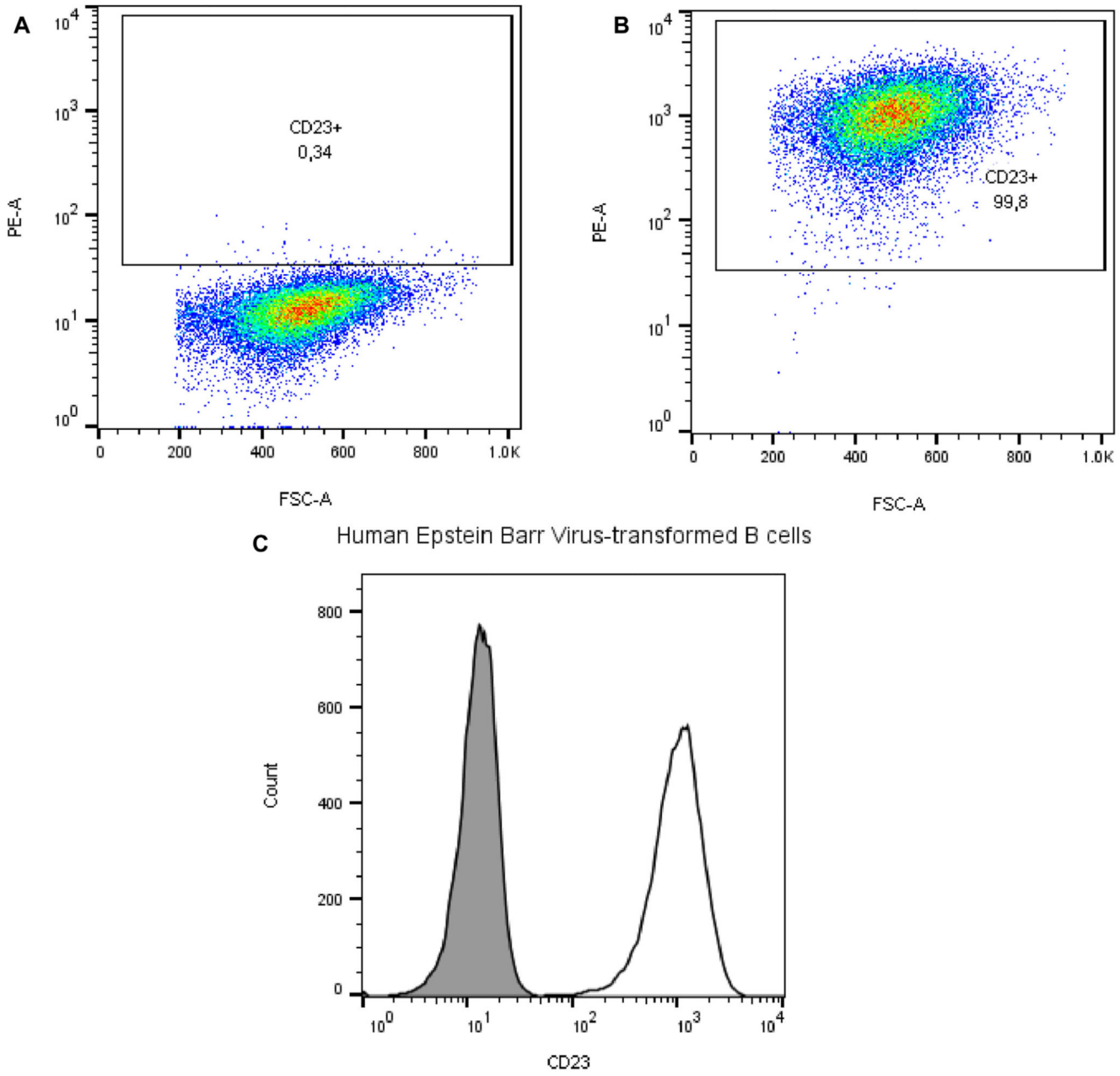

Fig E1.

CD23 expression on the EBV-transformed B-cell line used in the experiments. **A**, Dot plot representation of the staining of the isotype control (mouse IgG_1-phycoerythrin) on the EBV B cells. **B**, Dot plot representation of staining of CD23 on EBV B cells. **C**, Histogram representation of CD23 staining. The *white histogram* corresponds to CD23 staining, and the *gray histogram* represents the isotype control.

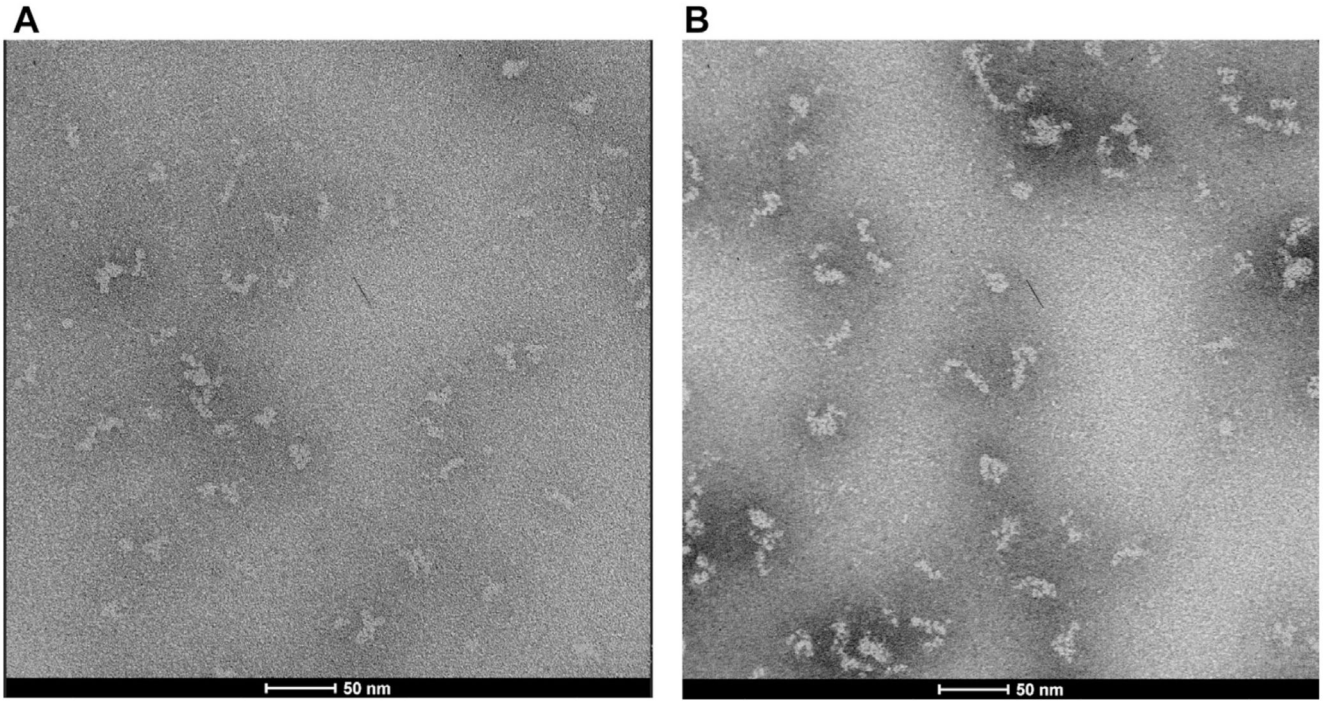

Fig E2.

Negative staining electron microscopic images of CB1 IgE–Bet v 1 complexes. CB1 IgE–Bet v 1 monomer complexes (**A**) and CB1 IgE–Bet v 1 oligomer complexes (**B**) are shown. *Bars* represent 50 nm.

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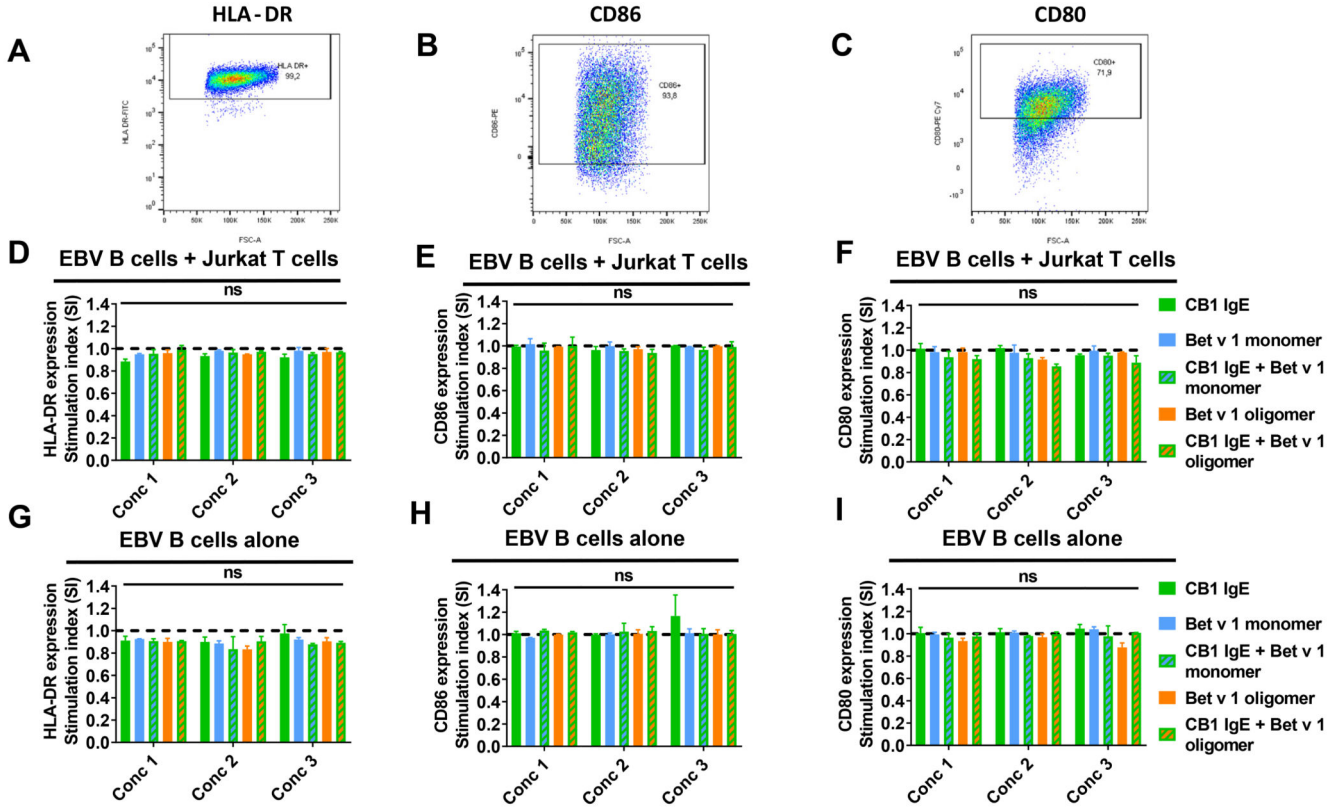

Fig E3.

HLA-DR, CD86, and CD80 expression on the human EBV-transformed B-cell line on incubation with various agents. **A-C**, Dot plot representation of HLA-DR (Fig E3, *A*), CD86 (Fig E3, *B*), and CD80 (Fig E3, *C*) expression on the surfaces of unstimulated human EBV-transformed B cells. **D-F**, HLA-DR (Fig E3, *D*), CD86 (Fig E3, *E*), and CD80 (Fig E3, *F*) expression on human EBV-transformed B cells expressed as MFI (*y-axes*; *dotted horizontal lines*) stimulated with 3 dilutions of different agents (*x-axes*: *Conc 1*, 26 nmol/L CB1 IgE alone or complexed with 294 nmol/L Bet v 1 monomer or oligomer in two 5-fold dilution steps [ie, *Conc 2* and *Conc 3*]) when subsequently coincubated with Jurkat T cells for 6 hours at 37°C. **G-I**, HLA-DR (Fig E3, *G*), CD86 (Fig E3, *H*), and CD80 (Fig E3, *I*) expression on human EBV-transformed B cells (*y-axes*; *dotted horizontal lines*) stimulated with 3 concentrations of agents (*Conc 1*: 26 nmol/L CB1 IgE alone or complexed with 294 nmol/L Bet v 1 monomer or oligomer in two 5-fold dilution steps [ie, *Conc 2* and *Conc 3*]) and subsequently coincubated in medium for 6 hours at 37°C. Each experiment was repeated at least twice in duplicates, and 1 representative result is displayed. *Bars* represent means ± SDs. *ns*, Nonsignificant, 2-way ANOVA.

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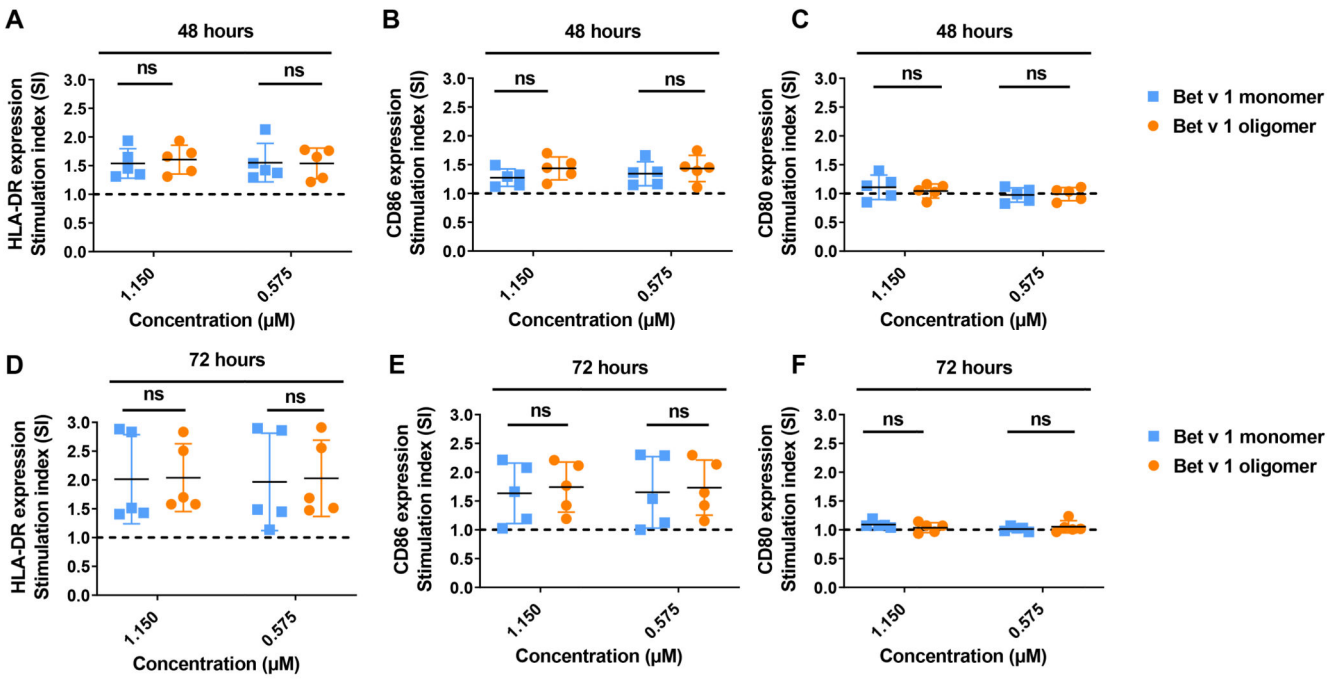

Fig E4.

HLA-DR, CD86, and CD80 expression on B cells from 5 patients with birch pollen allergy. Shown is HLA-DR (**A** and **D**), CD86 (**B** and **E**), and CD80 (**C** and **F**) expression on the surfaces of B cells ($CD19^+$) expressed as MFIs for HLA-DR and CD80 and percentage increases in $CD86^+$ cells for CD86 (*y-axes*; *dotted horizontal lines*) after 48 (Fig E4, *A-C*) or 72 (Fig E4, *D-F*) hours of incubation with Bet v 1 monomer (blue) or Bet v 1 oligomer (orange). Every condition in each patient was tested in duplicates. Data points represent individual values of the mean of the measurement for each patient and condition analyzed. Mean values ± SDs are also represented. *ns*, Nonsignificant, 2-way ANOVA.

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We thank Alina Neunkirchner for having generated the allergen-specific T-cell line and help with setting up the T-cell activation assay.

Abbreviations used

APC	Allophycocyanin
CB1 IgE	Chimeric Bip 1 IgE
CT Violet	CellTrace Violet cell proliferation kit
FACS	Fluorescence-activated cell sorting
FAP	Facilitated allergen presentation

IMDM	Iscove modified Dulbecco medium
MFI	Mean fluorescence intensity
RBL	Rat basophilic leukemia
TCR	T-cell receptor

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Key messages

- The ability of allergen-IgE complexes to cross-link CD23 governs the extent of CD23-mediated T-cell activation.
- The concentration of allergen-specific IgE controls specific T-cell activation in IgE-FAP.

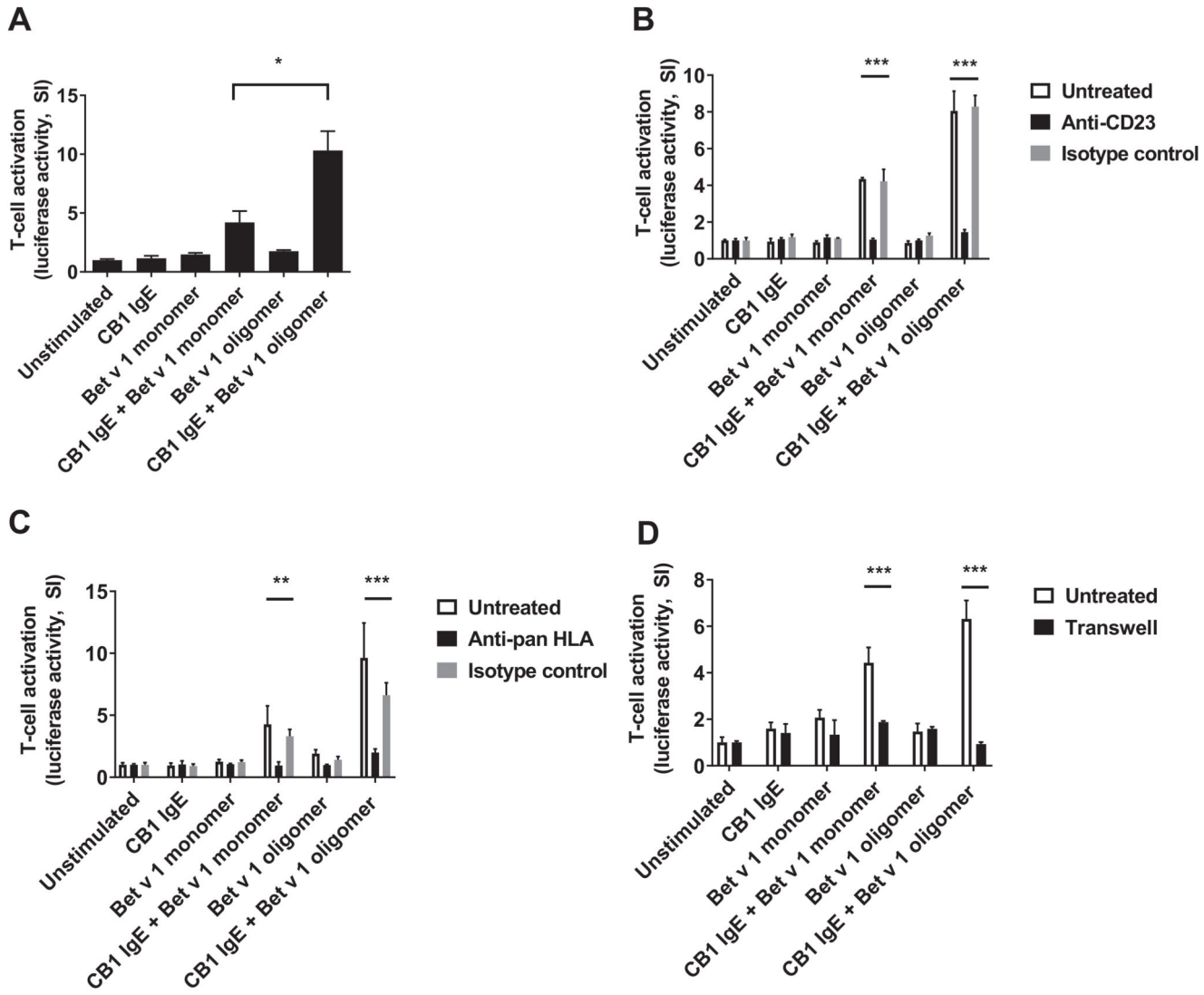

Fig 1.

A, Activation of Bet v 1–specific T cells by Bet v 1 or Bet v 1–IgE complexes presented by CD23-expressing EBV-transformed B cells. Activation of T cells (*y-axis*) exposed to different agents (*x-axis*) is shown. **B**, Dependence of IgE-FAP on CD23. T-cell activation (*y-axis*) in the presence of EBV-transformed B cells incubated with anti-CD23 antibody (black), isotype control (gray) or untreated (white), followed by addition of various agents (*x-axis*), is shown. **C**, Dependence of IgE-FAP on HLA. T-cell activation (*y-axis*) in the presence of EBV-transformed B cells incubated with anti–pan-HLA antibody (black), isotype control (gray) or untreated (white), followed by addition of various agents (*x-axis*), is shown. **D**, Dependence of IgE-FAP on cell-to-cell contact. T-cell activation (*y-axis*) in the presence of EBV-transformed B cells incubated with various agents (*x-axis*) and subsequently cocultured mixed with T cells (white) or separated by a transwell membrane (black) is shown. Each experiment was done at least twice in triplicates. One representative

result is displayed. *Bars* represent means ± SDs. *SI*, Stimulation index. **P* < .05, unpaired Student *t* test in Fig 1, *A*; ***P* < .01, ****P* < .001, 2-way ANOVA in Fig 2, *B-D*.

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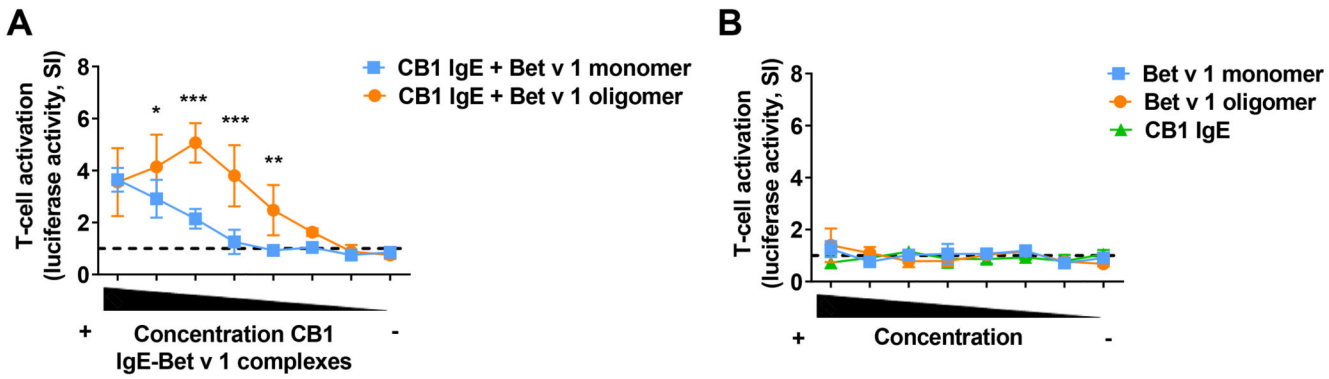

Fig 2.

T-cell activation induced by titrated concentrations of different Bet v 1–IgE complexes. **A**, T-cell activation (*y-axis*; SI = 1,*dotted horizontal line*) induced by titrated concentrations of CB1 IgE plus Bet v 1 monomer (blue) or CB1 IgE plus Bet v 1 oligomer (orange; 26 nmol/L CB1 IgE plus 294 nmol/L Bet v 1 monomer/oligomer in 5-fold dilution steps) presented by CD23-expressing human EBV-transformed B cells. **B**, T-cell activation (*y-axis*; SI = 1,*dotted horizontal line*) induced by titrated concentrations of Bet v 1 monomer (blue), Bet v 1 oligomer (orange), or CB1 IgE (green; 26 nmol/L CB1 IgE and 294 nmol/L Bet v 1 monomer/oligomer in 5-fold dilution steps). Each experiment was done at least twice in triplicates, and 1 representative result is displayed. *Bars* represent means ± SDs. *SI*, Stimulation index. **P* < .05, ***P* < .01, and ****P* < .001, 2-way ANOVA.

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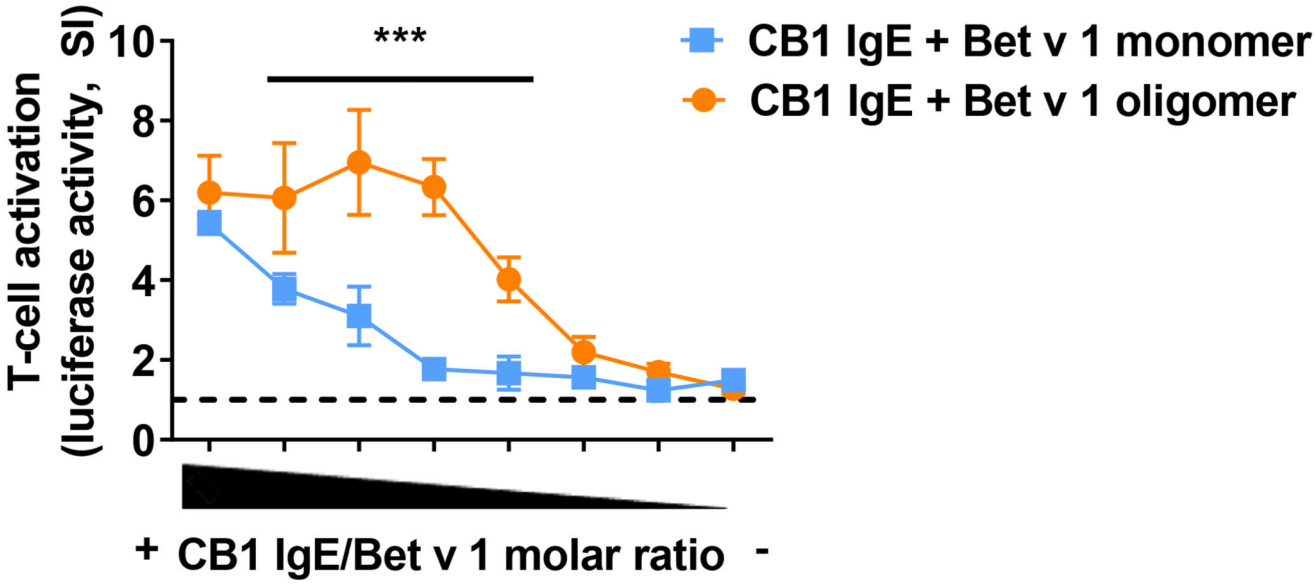

Fig 3.

CD23-mediated allergen presentation and subsequent T-cell activation increases with greater IgE concentrations. T-cell activation (*y-axis*; SI = 1, *dotted horizontal line*) induced by titrated concentrations of CB1 IgE plus Bet v 1 monomer (blue) or CB1 IgE plus Bet v 1 oligomer (orange; 26 nmol/L CB1 IgE in 5-fold dilution steps plus a constant concentration of 58.8 nmol/L Bet v 1 monomer/oligomer) is shown. Each experiment was done at least twice in triplicates, and 1 representative result is displayed. *Bars* represent means ± SDs. *SI*, Stimulation index. ***$P < .001$, 2-way ANOVA.

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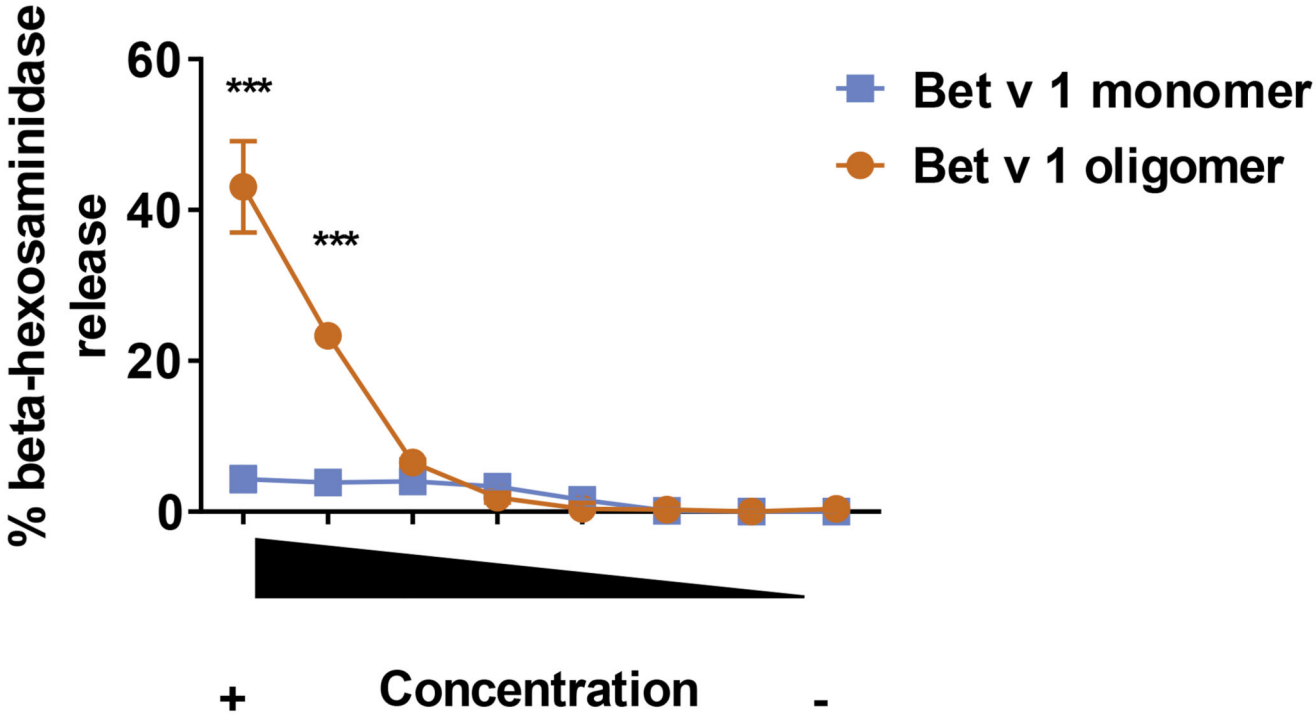

Fig 4.

Bet v 1 oligomer, but not Bet v 1 monomer, cross-links FcεRI-bound monoclonal CB1 IgE. Shown is the release of β-hexosaminidase (*y-axis*) induced from RBL cells expressing human FcεRI, which had been loaded with CB1 IgE and then stimulated with different equimolar concentrations of Bet v 1 monomer (blue) or oligomer (orange; *x-axis*: 294 nmol/L diluted in 5-fold steps). Each experiment was done at least twice in triplicates, and 1 representative result is displayed. *Bars* represent means ± SDs. ***$P < .001$, 2-way ANOVA.

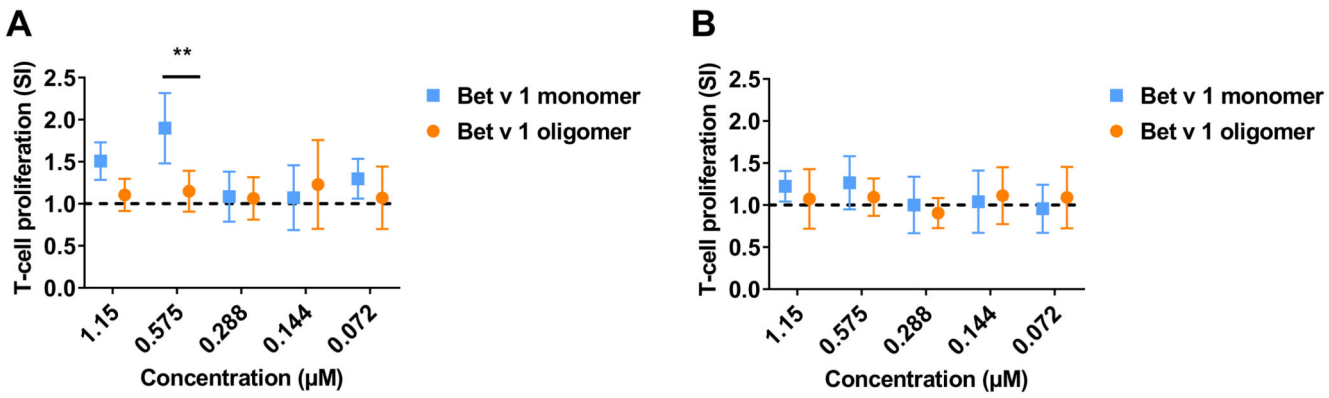

Fig 5.

Bet v 1 monomer is more efficient in inducing CD23-dependent T-cell proliferation than Bet v 1 oligomer. T-cell proliferation (*y-axes*; SI = 1, *dotted horizontal lines*) in PBMCs with (**A**) and without (**B**) B cells from 5 patients with Bet v 1 allergy stimulated with different equimolar concentrations of Bet v 1 monomer (blue) or oligomer (orange; *x-axes*). Every condition in each patient was tested in triplicates, and data points represent means of the median values of those triplicates. *Dots* represent means ± SDs (n = 5). Significant differences between Bet v 1 monomer and oligomer are shown. *SI*, Stimulation index. ** $P < .01$, 2-way ANOVA.

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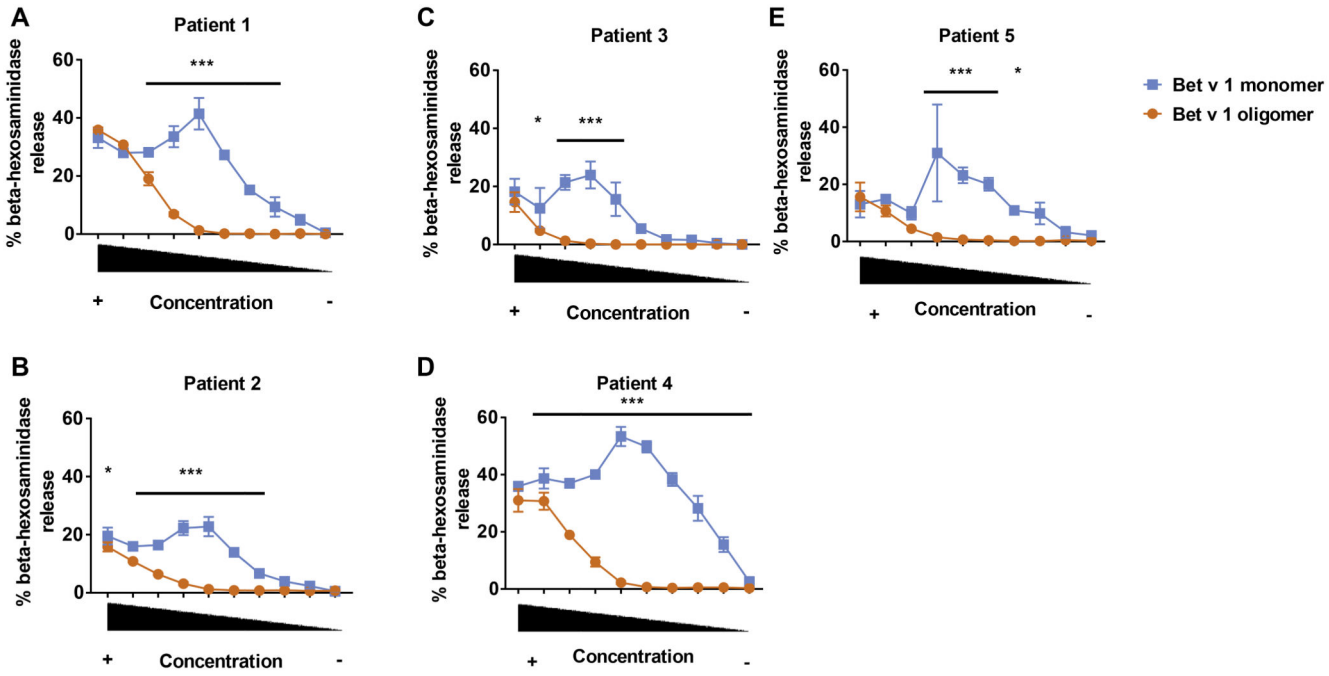

Fig 6.

Bet v 1 monomer cross-links FcεRI-bound allergic patients' polyclonal IgE more efficiently than Bet v 1 oligomer. Shown is the release of β-hexosaminidase (*y-axes*) induced from RBL cells expressing human FcεRI, which had been loaded with polyclonal IgE from 5 patients with birch pollen allergy (**A**, patient 1; **B**, patient 2; **C**, patient 3; **D**, patient 4; and **E**, patient 5) and were then stimulated with different equimolar concentrations of Bet v 1 monomer (blue) or oligomer (orange; *x-axes*: 23.53 nmol/L diluted in 4-fold steps). Data points shown for each patient represent means ± SDs (n = 3). Significant differences between Bet v 1 monomer and oligomer are shown. **P* < .05, ***P* < .01, and ****P* < .001, 2-way ANOVA.

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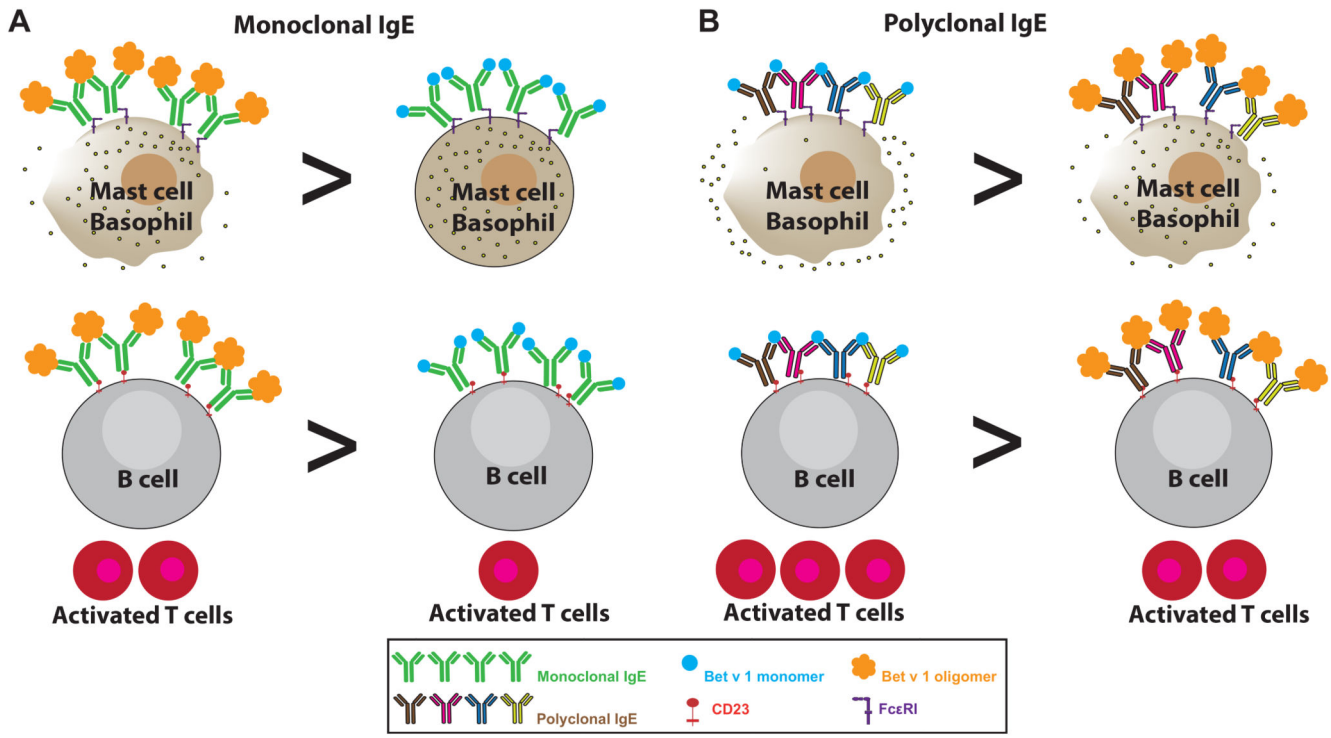

Fig 7.

Schematic representation of effector cell (ie, basophil and mast cell; *upper part*) and B-cell (*lower part*) activation and consecutive FAP by Bet v 1 monomer and Bet v 1 oligomer, depending on loading of cells with monoclonal (**A**) or polyclonal (**B**) IgE.

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Table 1

Patients' characteristics

Patient no.	Sex	Age (y)	Current birch pollen symptoms*	Other allergen sources†	Total IgE (kU/L)	Specific Bet v 1 IgE (kU_A/L)	Specific Bet v 1 IgE (%)	HLA-DR genotype
1	F	48	C, R	a, t	217.0	100.0	46.08	DRB1*03:01/ DRB1*04:04
2	F	25	C, R	h, t	198.0	103.0	52.02	DRB1*07:01/ DRB1*14:01
3	F	30	C, R	a, w	37.5	27.4	73.07	DRB1*11:03/ DRB1*15:01
4	M	35	C, R	a, h, t, w	333.0	169.0	50.75	DRB1*01:02/ DRB1*11:03
5	F	24	C, D, R	t, w	56.7	25.2	44.44	DRB1*07:01/ DRB1*11:04

F, Female; *M*, male; kU_A, kilounits of antigen.

**C*, Conjunctivitis; *D*, dermatitis; *R*, rhinitis.

†a, Animal dander; *h*, house dust mite; *t*, tree pollen (other than birch); *w*, weed pollen.