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REFERENCES

- Hiragun M, Hiragun T, Ishii K, Suzuki H, Tanaka A, Yanase Y, et al. Elevated serum IgE against MGL_1304 in patients with atopic dermatitis and cholinergic urticaria. *Allergol Int* 2014;63:83-93.
- Metz M, Altrichter S, Ardelean E, Kessler B, Krause K, Magerl M, et al. Anti-immunoglobulin E treatment of patients with recalcitrant physical urticaria. *Int Arch Allergy Immunol* 2011;154:177-80.
- Metz M, Bergmann P, Zuberbier T, Maurer M. Successful treatment of cholinergic urticaria with anti-immunoglobulin E therapy. *Allergy* 2008;63:247-9.
- Maurer M, Metz M, Brehler R, Hillen U, Jakob T, Mahler V, et al. Omalizumab treatment in patients with chronic inducible urticaria: a systematic review of published evidence. *J Allergy Clin Immunol* 2018;141:638-49.
- Sigler RW, Levinson AI, Evans R III, Horakova Z, Kaplan AP. Evaluation of a patient with cold and cholinergic urticaria. *J Allergy Clin Immunol* 1979;63:35-8.
- Asady A, Ruft J, Ellrich A, Hawro T, Maurer M, Altrichter S. Cholinergic urticaria patients of different age groups have distinct features. *Clin Exp Allergy* 2017;47:1609-14.
- Ghazanfar MN, Sand C, Thomsen SF. Effectiveness and safety of omalizumab in chronic spontaneous or inducible urticaria: evaluation of 154 patients. *Br J Dermatol* 2016;175:404-6.
- Metz M, Ohanyan T, Church MK, Maurer M. Omalizumab is an effective and rapidly acting therapy in difficult-to-treat chronic urticaria: a retrospective clinical analysis. *J Dermatol Sci* 2014;73:57-62.
- Fukunaga A, Washio K, Hatakeyama M, Oda Y, Ogura K, Horikawa T, et al. Cholinergic urticaria: epidemiology, pathophysiology, new categorization, and management. *Clin Auton Res* 2018;28:103-13.

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Identification of bee venom Api m 1 IgE epitopes and characterization of corresponding mimotopes

To the Editor:

Honeybee venom allergy is potentially life-threatening for allergic subjects because a single sting may cause fatal anaphylaxis. Although immunotherapy with bee venom extract is well established, high rate of side effects (from 16% up to 40%),¹ high percentage of relapses (from 15% to 20%), and treatment duration (3-5 years)² prompt new immunotherapeutic strategies. Vaccination based on B-cell epitopes³ offers a promising approach that has not yet been studied in honeybee venom allergy. With the sensitivity of up to 97%,⁴ the major bee venom allergen Api m 1 is the most suitable for generation of epitope-based immunotherapeutics. In this study, we identified IgE epitopes and characterized mimotopes of Api m 1.

Full methodological details are provided in this article's Online Repository at www.jacionline.org. The biopanning of phage display libraries with anti-Api m 1 IgG yielded 46 unique peptides. Most of the peptides exhibited high and selective binding to the target antibodies and competed with native

Api m 1 (nApi m 1) for the same paratopes (see Fig E1, A and B, in this article's Online Repository at www.jacionline.org).

The obtained peptide sequences were arranged into 2 groups according to the consensus motifs. The first group of 29 peptides from linear libraries and 7 peptides from cyclic library carrying GP[S/N/D]ELG[R/K/H] motif aligned with the sequence region G₁₇P₁₈N₁₉E₂₀L₂₁G₂₂R₂₃F₂₄ of Api m 1 (Fig 1, A). The second group of 10 peptides from cyclic library carrying D[K/R/H]SKP(H) motif aligned with the D₁₁₉K₁₂₀S₁₂₁K₁₂₂P₁₂₃K₁₂₄ sequence of Api m 1 (Fig 1, B). The peptides were further mapped onto the 3-dimensional structure of Api m 1 using computer-based epitope mapping program Pepitope. Conformational mapping substantially overlapped with primary sequence alignment, indicating that the regions 17-24 and 119-124 represent the epitopes of Api m 1 (Fig 1, C, red and green, respectively). Importantly, both defined epitopes are located on the solvent-exposed loops protruding from the allergen surface. This is one of the main traits of antibody epitopes and was also characteristic of IgE-binding sites on Api m 2⁵ and Ves v 5.⁶

For use in immunotherapy, short peptides need to be coupled to a carrier that provides T-cell help for induction of antibody response because they are not immunogenic by themselves. Bacteriophages demonstrated good potential as peptide carriers in animal models.⁷ However, their clinical use is limited because of the fast clearance after parenteral application and strict regulatory requirements. Furthermore, multivalent display of mimotopes on phage may cause IgE cross-linking and activation of effector cells. Therefore, we propose the use of minor coat protein pIII from M13 bacteriophage alone for a carrier. pIII maintains the correct conformation of mimotopes, ensuring their appropriate presentation to the immune cells. Because the peptides showed similar binding to the target antibodies, 4 randomly selected representatives (linear [SPPNALGRFLPD, LMGPSEL] and cyclic [CWTDLGRKC, CVDKSKPHC]) were chosen for further characterization.

Given that antibodies of IgG isotype were used as target, the reactivity of selected peptides with patients' IgE was tested to determine whether they are also mimetics of IgE epitopes. Sera from 12 honeybee venom-allergic patients (patients 1-12; see Table E1 and patient characteristics in this article's Online Repository at www.jacionline.org) showed IgE binding to the 4 selected pIII-fused peptides (isolated from *E coli* periplasm), which was stronger than binding to nApi m 1 at equimolar concentrations (Fig 1, D). This means that the peptides were better exposed on pIII than on nApi m 1, thus demonstrating beneficial display properties of pIII carrier. Because IgE to Hymenoptera venoms are common in general population,⁸ sera from healthy subjects could not serve as negative controls. Instead, 2 unrelated pIII-fused control peptides were used and showed no reactivity (Fig 1, D). Hence, binding was attributed only to the peptides that mimic the IgE epitopes of Api m 1 (mimotopes). Thus, the mimotopes bound to patients' IgE as well as to target IgG, which indicates that the identified epitopes are relevant for both antibody isotypes. However, because the mimotopes were selected by IgG antibodies, additional IgE epitopes on Api m 1 cannot be excluded.

Four synthetic mimotopes were evaluated for allergenic activity and T-cell response-modulating potential in 3 patients (patients 1-3; see Table E1). One carrier-bound mimotope's representative (randomly selected pIII-fused CVDKSKPHC) was tested to assess the impact of pIII carrier on basophil

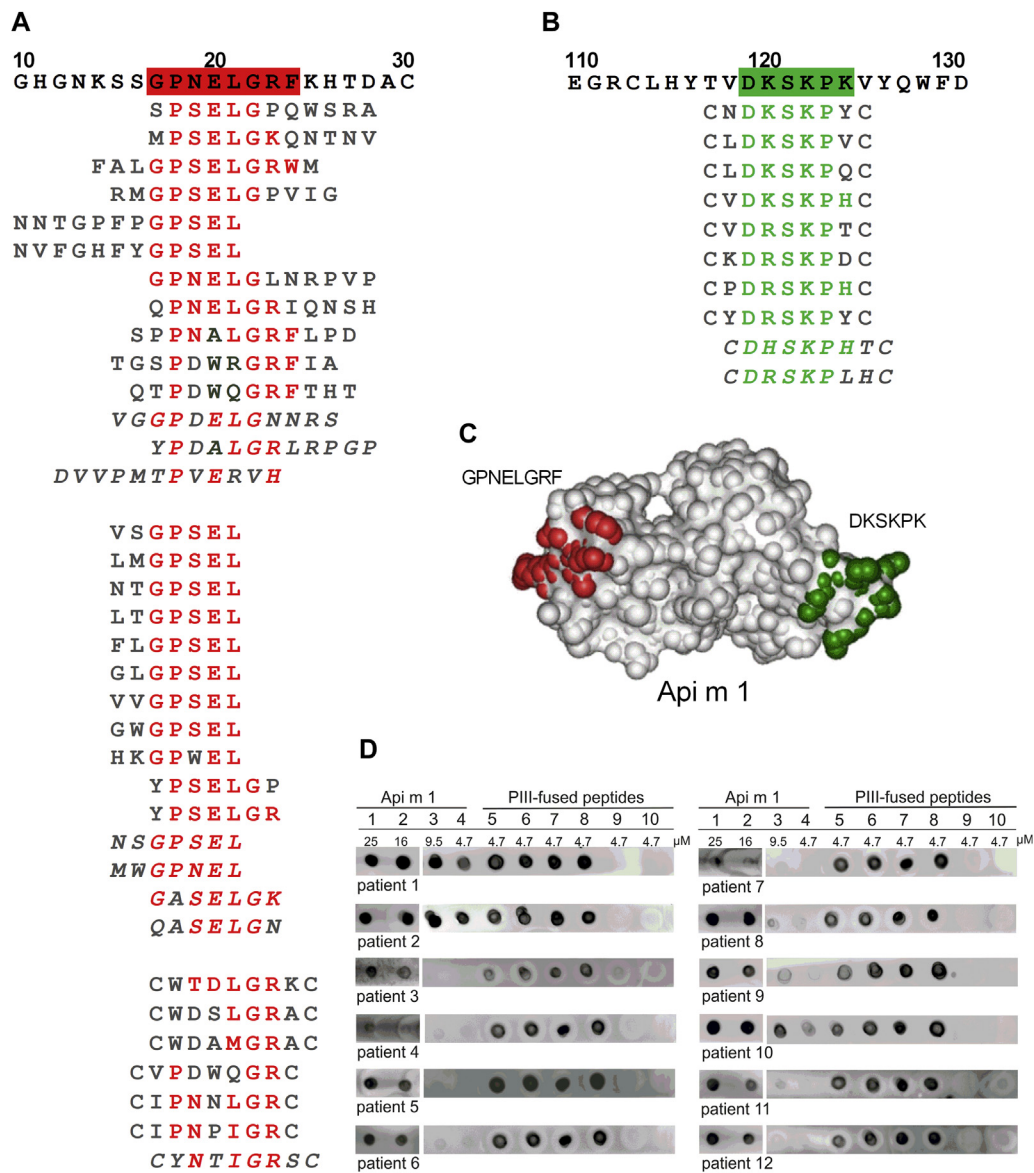


FIG 1. The peptide sequences obtained by biopanning of phage display libraries with anti-Api m 1 IgG. **A**, Peptides carrying consensus motif GP[S/N/D]ELG[R/K/H] were aligned to the N-terminal region of Api m 1. **B**, Peptides carrying D[K/R/H]SKP(H) consensus motif were aligned to the C-terminal region of Api m 1. Amino acids in red (**A**) and green (**B**) are the same or have similar physicochemical properties as residues in Api m 1 primary sequence. **C**, The surface locations of the 2 epitopes (red and green) identified by computational mapping of mimotopes to the 3-dimensional structure of Api m 1 are presented. **D**, Binding of nApi m 1 and pIII-fused peptides to IgE from sera of bee venom-allergic patients. Increasing concentration of nApi m 1 (1-4). Peptides matching to Api m 1 epitope between residues 17 and 24: pIII-SPPNALGRFLPD (5), pIII-LMGPSSEL (6), and pIII-CWTDLGRKC (7). Peptide matching to Api m 1 epitope between residues 119 and 124: pIII-CVDKSKPHC (8). Control peptides: pIII-GTFDHPQ binding streptavidin (9) and pIII-CRMQMPSDC binding antihuman leptin antibodies (10).

activation and cytokine production. In response to bee venom and nApi m 1, basophils of all 3 patients showed upregulated CD63 (Fig 2, A and B), whereas pIII-fused mimotope CVDKSKPHC induced no relevant CD63 expression (Fig 2, C). Synthetic mimotopes retained conformation in solution and did not cause IgE-independent activation of basophils (see Fig E1, C, and Fig E2, A, in this article's Online Repository at www.jacionline.org).

There was no difference in basophil activation between synthetic and corresponding pIII-fused mimotope. Therefore, pIII carrier had no impact on allergenic activity.

T-cell response-modulating potential of mimotopes was assessed by measuring secretion of T_H2-specific (IL-5, IL-13) and T_H1-specific (IL-2, IFN-γ) cytokines in cultures of PBMCs. Stimulation of cultures with bee venom and nApi m 1 elevated

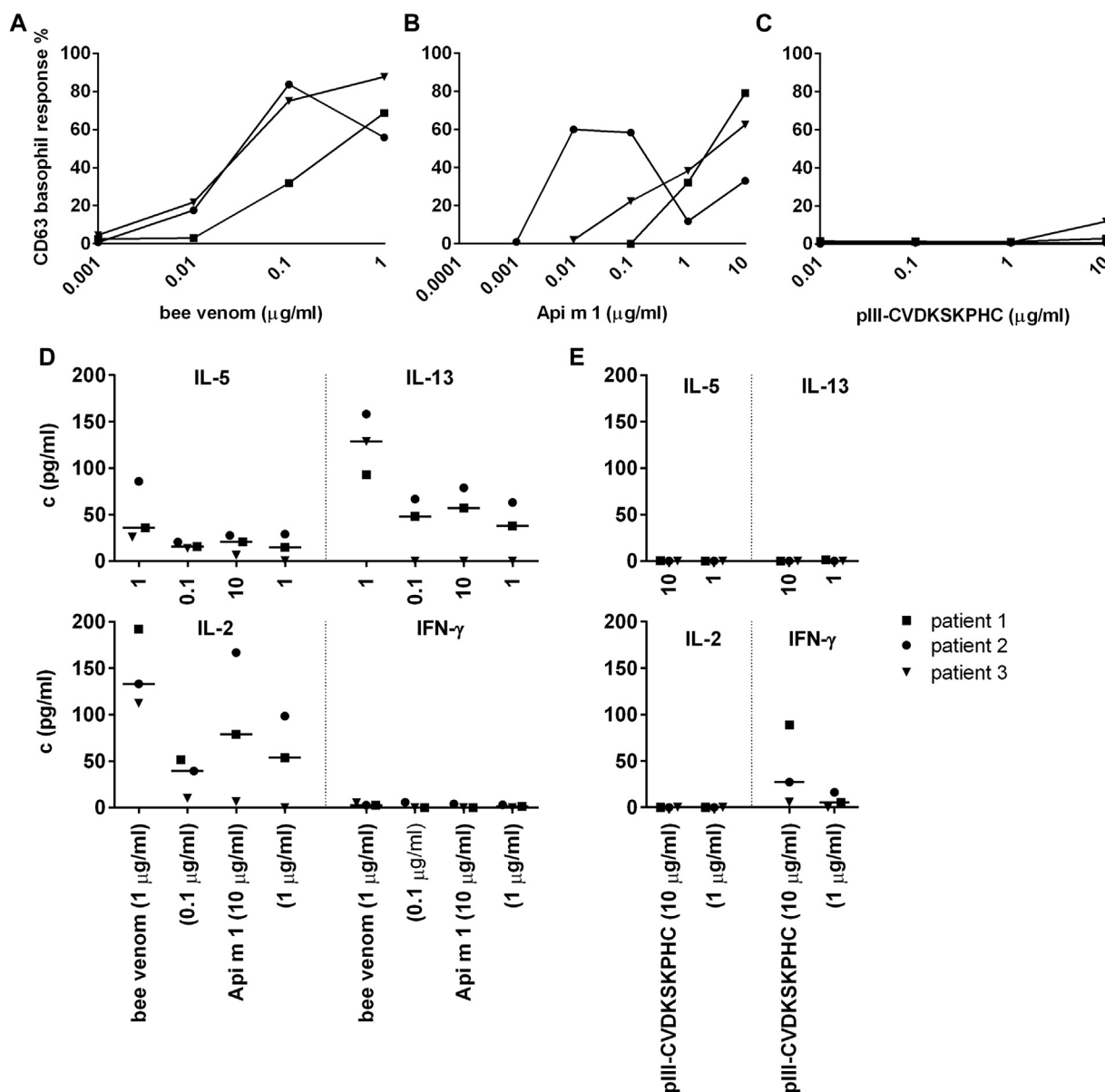


FIG 2. Basophil response determined by the percentage of CD63-expressing cells in bee venom-allergic patients (patients 1-3). **A**, After addition of bee venom. **B**, After addition of nApi m 1. **C**, After addition of representative pIII-fused peptide mimotope. Cytokines production after 48-hour stimulation of PBMCs in bee venom-allergic patients (patients 1-3). **D**, With bee venom and nApi m 1. **E**, With representative pIII-fused peptide mimotope. The middle lines represent median.

the levels of IL-5, IL-13, and IL-2 (Fig 2, D). Synthetic mimotopes elicited negligible cytokine secretion (see Fig E2, B), whereas pIII-fused mimotope CVDKSKPHC induced the production of IFN-γ (Fig 2, E). This suggests potential shift from T_H2 toward T_H1 pattern and might imply that pIII carrier is capable of eliciting T_H1 immune response that was found during bee venom immunotherapy.⁹

In conclusion, this is the first time that the IgE epitopes of Api m 1 have been identified. They do not include glycosyl moiety, which substantiates recent studies reporting no influence of carbohydrate moiety on allergenicity of Api m 1. Our work contributes to better understanding of mechanisms involved in the pathogenesis of sting anaphylaxis and can further improve diagnosis and prognosis of bee venom allergy. Corresponding

mimotopes represent novel candidates for the development of more precise and safer allergen immunotherapy.

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REFERENCES

1. Dhimi S, Zaman H, Varga EM, Sturm GJ, Muraro A, Akdis CA, et al. Allergen immunotherapy for insect venom allergy: a systematic review and meta-analysis. *Allergy* 2017;72:342-65.
2. Ruëff F, Vos B, Oude Elberink J, Bender A, Chatelain R, Dugas-Breit S, et al. Predictors of clinical effectiveness of Hymenoptera venom immunotherapy. *Clin Exp Allergy* 2014;44:736-46.
3. Niederberger V, Neubauer A, Gevaert P, Zidarn M, Worm M, Aberer W, et al. Safety and efficacy of immunotherapy with the recombinant B-cell epitope-based grass pollen vaccine BM32. *J Allergy Clin Immunol* 2018;142:497-509.
4. Müller UR, Johansen N, Petersen AB, Fromberg-Nielsen J, Haeberli G. Hymenoptera venom allergy: analysis of double positivity to honey bee and *Vespa* venom by estimation of IgE antibodies to species-specific major allergens Api m1 and Ves v5. *Allergy* 2009;64:543-8.
5. Padavattan S, Schirmer T, Schmidt M, Akdis C, Valenta R, Mittermann I, et al. Identification of a B-cell epitope of hyaluronidase, a major bee venom allergen, from its crystal structure in complex with a specific Fab. *J Mol Biol* 2007;368:742-52.
6. dos Santos-Pinto JR, dos Santos LD, Arcuri HA, da Silva Menegasso AR, Pego PN, Santos KS, et al. B-cell linear epitopes mapping of antigen-5 allergen from *Polybia paulista* wasp venom. *J Allergy Clin Immunol* 2015;135:264-7.
7. Sturm GJ, Schuster C, Kranzelbinder B, Wiednig M, Groselj-Strele A, Aberer W. Asymptomatic sensitization to hymenoptera venom is related to total immunoglobulin E levels. *Int Arch Allergy Immunol* 2009;148:261-4.
8. Henry KA, Arbabi-Ghahroudi M, Scott JK. Beyond phage display: non-traditional applications of the filamentous bacteriophage as a vaccine carrier, therapeutic biologic, and bioconjugation scaffold. *Front Microbiol* 2015;6:755.
9. Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Müller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN- γ secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995;154:4187-94.

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Imatinib stimulates prostaglandin E₂ and attenuates cytokine release via EP4 receptor activation



To the Editor:

Imatinib is a highly efficacious therapy for leukemias that are characterized by the presence of the Philadelphia chromosome, a reciprocal translocation that fuses the genes encoding breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homolog (ABL) resulting in the expression of the constitutively active protein kinase BCR-ABL. In addition, imatinib was shown to affect immune responses; for instance, beneficial effects have been described in rheumatoid arthritis¹ and asthma.²

In human peripheral blood monocytes and in macrophages, imatinib inhibits LPS-induced production of TNF- α , IL-6, and IL-8, via inhibition of nuclear factor kappa B (NF- κ B).³ Because imatinib shows anti-inflammatory and immunomodulatory effects, we were interested in its effect on prostanoid synthesis, especially on prostaglandin (PG)E₂. Prostaglandins (PGs) and thromboxane A₂ (TXA₂) are formed from arachidonic acid, which is released by cytosolic phospholipase c A₂ from membrane phospholipids. Arachidonic acid is metabolized by cyclooxygenases to the unstable intermediate PGH₂, which is

further converted by the respective terminal synthases to PGE₂, PGI₂, PGD₂, PGF_{2 α} , and TXA₂. PGE₂ is one of the most abundant PGs and exerts its effects by binding to 4 different G protein-coupled receptors (EP1-EP4), which show tissue-specific distribution. PGE₂ was shown to inhibit immune responses mainly via EP4 activation, by curbing cytokine and chemokine expression and promoting a shift of the T_H1 to a T_H2 response, although the EP2 receptor has similar and redundant roles.⁴ Thus, we hypothesized that the inhibitory effect of imatinib on proinflammatory cytokines might be mediated by PGE₂ via EP4 receptor activation. The study was approved by the Ethics Committee of the Medical University of Graz to withdraw blood of healthy donors (17-291 ex 05/06) and from patients with chronic myeloid leukemia in complete remission, receiving imatinib (28-590 ex 15/16), after signing informed consent (for patients' data, see Table E1 and Fig E1 in this article's Online Repository at www.jacionline.org; also see this article's Methods section in the Online Repository at www.jacionline.org).

First, we investigated the effect of imatinib *in vitro*. In adherent human monocytes treated with LPS (10 ng/mL) and further incubated for 20 hours, we found that imatinib caused a significant inhibition of TNF- α that was paralleled by a pronounced (5-10-fold) increase in PGE₂ levels and interestingly an inhibition of TXA₂, measured as its stable metabolite TXB₂ (Fig 1, A-C). Pretreatment with diclofenac (1 μ M), which totally inhibited PGE₂ synthesis, and the selective EP4 receptor antagonist ONO-AE3 208 (0.3 μ M) counteracted the inhibitory effect of imatinib on TNF- α release, underscoring that this effect is indeed mediated by PGE₂-induced EP4 receptor activation (Fig 1, G and H), whereas antagonism of the EP2 as well as EP1 and EP3 receptors had no effect (see Fig E2 in this article's Online Repository at www.jacionline.org). This line of reasoning is further strengthened by the fact that the imatinib-induced increase in PGE₂ precedes the inhibition of TNF- α formation (Fig 1, D-F). The same results were obtained in human monocyte-derived macrophages and human whole blood (see Figs E3 and E4 in this article's Online Repository at www.jacionline.org). Most importantly, we observed the same effects also after treatment with another BCR-ABL inhibitor, nilotinib (see Figs E3-E5 in this article's Online Repository at www.jacionline.org).

In addition, we could prove that the inhibitory effect of imatinib on LPS-stimulated (100 ng/mL) NF- κ B activation is dependent on PGE₂ and EP4 receptor activation, as shown in the human macrophage cell line U937 containing an NF- κ B-GFP reporter cassette⁵ (Fig 1, I). This observation further corroborates the hypothesis that PGE₂ via EP4 receptor activation inhibits NF- κ B, which leads to reduced cytokine production. Data in the Online Repository show that upstream kinases were not involved in the TNF- α inhibition by imatinib and thus further support the notion of an essential mediator role of PGE₂ and EP4 (see Fig E6 in this article's Online Repository at www.jacionline.org). In washed platelet preparations and platelet microsomes we provide evidence that imatinib and nilotinib inhibit arachidonic acid-induced thromboxane synthase (or in case of microsomes PGH₂); however, because accumulating PGH₂ acts on thromboxane receptors, platelet aggregation is not affected (see data in this article's Online Repository at www.jacionline.org; also see Fig E7 in this article's Online Repository at www.jacionline.org).

METHODS

Biopanning of phage display libraries

Affinity-purified rabbit anti-Api m 1 IgG (MyBioSource, San Diego, Calif, Cat# MBS573431, RRID: AB_2716318) were used as target. Their binding to Api m 1 (Bee venom phospholipase A2, Latoxan, Valence, France) was checked by direct ELISA using horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (Thermo Fisher Scientific, Waltham, Mass, Cat# G-21234 RRID: AB_2536530) for detection. Because the IgG isotype of target antibodies was used in biopanning, the conserved epitope specificity between human IgE and the target antibodies was subsequently tested to determine whether the identified peptides are also mimotopes of patients' IgE. For biopanning, 3.6 µg of anti-Api m 1 antibodies was immobilized alternately onto 0.45 mg of protein G- or protein A-coupled magnetic beads (Dynabeads, Thermo Fisher Scientific) by incubation in 0.05% PBST for 30 minutes at room temperature. Linear heptamer, linear dodecamer, and cyclic heptamer random peptide phage display libraries (New England Biolabs, Ipswich, Mass) were screened according to manufacturer's instruction. Bound phages were eluted from the target antibodies either with 0.1 mol/L glycine-HCl (pH 2.2) for 10 minutes followed by immediate neutralization with 1 mol Tris (pH 8.0) or competitively with Api m 1 at the final concentration of 12 µg/mL. Phages were propagated in *Escherichia coli* host strain K12 ER2738 (New England Biolabs). Three binding/amplification cycles were performed. After the third round, 24 single-phage plaques for each elution method (total 144 clones) were randomly picked, amplified, and tested for reactivity with target antibodies in preliminary phage ELISA.

Preliminary phage ELISA

MaxiSorp microtiter plates (Thermo Fisher Scientific) were coated with 2 µg/mL of anti-Api m 1 IgG (RRID: AB_2716318) in PBS overnight at 4°C. Plates were blocked with 5% BSA in PBS for 1.5 hours at room temperature and washed 3 times with 0.1% PBST. A separate set of wells was covered with blocking buffer only, to determine background binding. The amplified phage clones or control (wild-type phage clone with no peptide displayed on its surface) in Luria-Bertani broth were then loaded into the wells. After 60 minutes of incubation, the wells were washed 5 times with 0.1% PBST. The detection was performed as described in Luzar et al^{E1} using HRP-conjugated anti-M13 mAbs (GE Healthcare, Chicago, Ill, Cat# 27942101, RRID: AB_2616587). A total of 59 clones with highest target to background absorbance ratio were subjected to DNA sequencing (GATC Biotech, Konstanz, Germany) to determine whether the selected peptides were converging on a particular consensus sequence.

Characterization of phage-displayed peptides binding to target antibodies

Forty-six peptide-displaying phage clones were purified by PEG-precipitation, resuspended in PBS, and quantified by spectrophotometry. All ELISA experiments for characterization of the phage-displayed peptides were performed as described for preliminary phage ELISA. The suspensions containing 2×10^9 pfu of individual phage clones or control phage in PBS were loaded into the wells. For testing a potential nonspecific binding to antibody constant region, wells were coated with rabbit antiserum (anti-Fel d 1, Indoor Biotechnologies, Cardiff, United Kingdom, Cat# PA-FD1, RRID: AB_2716556) diluted 1:1000 in PBS overnight at 4°C. In competition ELISA Api m 1 in 3 different concentrations (1, 5, 10 µg/mL) was added to the phage clones and incubated with target antibodies for 1 hour at room temperature.

Computational mapping of peptides to the surface of Api m 1

Amino acid sequences were examined using the MimoDB 2.0 database to exclude target-unrelated peptides and aligned to Api m1 primary sequence with Clustal Omega.^{E2} Pepsurf algorithm^{E3} was used to map peptides onto the 3-dimensional structure of Api m 1 (PDB ID 1POC). The surface locations of

the amino acids that represent epitopes were presented with molecular graphics visualization tool ViewerLite.

Isolation and enrichment of pIII-fused peptides from *E coli* periplasm

pIII-fused peptides were extracted from the periplasm of *E coli* ER2738. Two unrelated pIII-fused peptides (linear GTFDHPQ^{E4} and cyclic CRMQMPSDC^{E5}; targeting streptavidin and antihuman leptin antibodies, respectively) were used as controls. Host bacteria were infected with individual peptide-displaying phage clone and grown for 2 hours at 37°C with agitation. Bacterial pellets were spun down at 5000g for 10 minutes and resuspended in 1 mL of an ice-cold solution consisting of 20% sucrose, 200 mM Tris-HCl (pH 8.0), and 1 mM EDTA supplemented with protease inhibitor cocktail (EZBlock, BioVision, San Francisco, Calif) at a dilution of 1:200. After 1-hour incubation on ice with occasional stirring, supernatants were harvested by centrifugation at 12000g for 20 minutes at 4°C. The resulting periplasmic extracts were concentrated (4-5-fold), and the extraction buffer was exchanged for PBS by ultrafiltration with 10 kDa cutoff membrane (Microsep Advance Centrifugal Device, Pall Corporation, New York, NY). Enrichment of pIII-fused peptides from periplasmic extracts was performed with corresponding peptide targets immobilized onto beads. We used anti-Api m 1 IgG (RRID: AB_2716318) and antihuman leptin antibody (R&D Systems, Minneapolis, Minn, Cat# MAB398, RRID:AB_2136056) immobilized to Dynabeads M-280 tosylactivated (Thermo Fisher Scientific) according to manufacturer's protocol and streptavidin-coupled Dynabeads MyOne Streptavidin T1 (ThermoFisher Scientific). Coated beads were incubated with periplasmic extracts for 1 hour at room temperature and then washed 3 times with 0.01 mol Na-phosphate (pH 7.4). Finally, captured pIII-fused peptides were eluted in 25 µL glycine-HCl (pH 2.5) following immediate neutralization with 1 mol Tris (pH 8.0). The concentration of proteins was determined with Bradford assay.

Immunodot assay: Binding of pIII-fused peptides to patients' IgE

Two microliters of each sample containing 200 µg/mL (4.7 µM) of pIII-fused peptides was spotted onto 0.45 µm nitrocellulose membrane (GE Healthcare). Native Api m 1 (nApi m 1) was used as positive control. The membrane was blocked with 5% skimmed milk in Tris-buffered saline/0.05% Tween 20 (TBST) for 3 hours at room temperature and then incubated overnight at 4°C with patient serum diluted 1:10 in 0.05% TBST. Following triple washing with 0.1% TBST, membranes were incubated with HRP-conjugated goat antihuman IgE antibodies (Thermo Fisher Scientific, Cat# A18793, RRID: AB_2535570) diluted 1:2000 in 1% BSA/0.1% TBST for 2 hours at room temperature. The reactive dots were visualized with CCD image analysis system (G-Box, Syngene, United Kingdom) after 5-minute incubation in SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific).

Chemical synthesis of peptides

Peptides were synthesized by Innovagen (Lund, Sweden). To obtain stable cyclic peptides, cyclization was performed via amide bond by introducing additional glutamic acid and linking its side chain to the N-terminal end of the peptide. Negatively charged C-terminal carboxylate was amidated in all peptides. Peptides were purified by HPLC to greater than 90%. To demonstrate that the synthetic mimotopes adopt the same conformation in solution as phage-displayed mimotopes, competition ELISA was performed by incubating synthetic peptides with respective phage clones (1×10^{10} pfu) and analyzing binding to the target antibody as described in preliminary phage ELISA.

Basophil activation test

Whole-blood samples (patients 1-3, Table E1) were incubated with synthetic peptides (from 100 to 0.1 µg/mL), pIII-fused peptide CVDKSKPHC

(from 10 to 0.01 $\mu\text{g/mL}$), natural Api m 1 (nApi m 1; Latoxan, Valence, France; from 10 to 1.0×10^{-5} $\mu\text{g/mL}$), and bee venom (Hal Allergie, Haarlem, The Netherlands; from 1 to 0.001 $\mu\text{g/mL}$) for 15 minutes at 37°C. For control purposes, cells were exposed to 0.55 $\mu\text{g/mL}$ of anti-Fc ϵ RI mAb (Bühlmann Laboratories AG, Schönenbuch, Switzerland), 50 $\mu\text{g/mL}$ of fMLP (Sigma-Aldrich, St Louis, Mo), or stimulation buffer alone. Degranulation was stopped by transferring tubes on ice, after which fluorescein isothiocyanate-conjugated anti-CD63 mAb, phycoerythrin-conjugated anti-CD123 mAb, and PerCP-conjugated anti-HLA-DR mAb (BD Biosciences, San Jose, Calif) were added and incubated for 20 minutes at 4°C. Finally, whole-blood probes were lysed, washed, fixed, and analyzed within 2 hours on a FACSCanto II flow cytometer (BD Biosciences) as previously described.^{E6}

Cytokine measurements

PBMCs were isolated using Ficoll-Paque density gradient, washed twice with PBS, and resuspended in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin (all Sigma-Aldrich). Isolated PBMCs were seeded in final concentration of 2×10^5 cells per well and incubated for 48 hours at 37°C and 5% CO₂ with 1 to 0.1 $\mu\text{g/mL}$ of bee venom and 10 to 0.1 $\mu\text{g/mL}$ of nApi m 1, synthetic peptides, and pIII-fused peptide CVDKSKPHC each. Four cytokines, IL-2, IL-5, IL-13, and IFN- γ ,

were quantified in the supernatants with the Cytometric Bead Array (BD Biosciences) according to the manufacturer's protocol and analyzed on FACSCalibur flow cytometer (BD Biosciences). The results were normalized for heterogeneous background proliferation.

REFERENCES

- E1. Luzar J, Molek P, Šilar M, Korošec P, Košnik M, Štrukelj B, et al. Identification and characterization of major cat allergen Fel d 1 mimotopes on filamentous phage carriers. *Mol Immunol* 2016;71:176-83.
- E2. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 2011;7:539.
- E3. Mayrose I, Penn O, Erez E, Rubinstein ND, Shlomi T, Freund NT, et al. Pepitope: epitope mapping from affinity-selected peptides. *Bioinformatics* 2007;23:3244-6.
- E4. Lunder M, Bratkovic T, Doljak B, Kreft S, Urleb U, Štrukelj B, et al. Comparison of bacterial and phage display peptide libraries in search of target-binding motif. *Appl Biochem Biotechnol* 2005;127:125-31.
- E5. Molek P, Bratkovic T. Epitope mapping of mono- and polyclonal antibodies by screening phage-displayed random peptide libraries. *Acta Chim Slov* 2016;63: 914-9.
- E6. Korošec P, Erzen R, Šilar M, Bajrovic N, Kopac P, Kosnik M. Basophil responsiveness in patients with insect sting allergies and negative venom-specific immunoglobulin E and skin prick test results. *Clin Exp Allergy* 2009;39:1730-7.

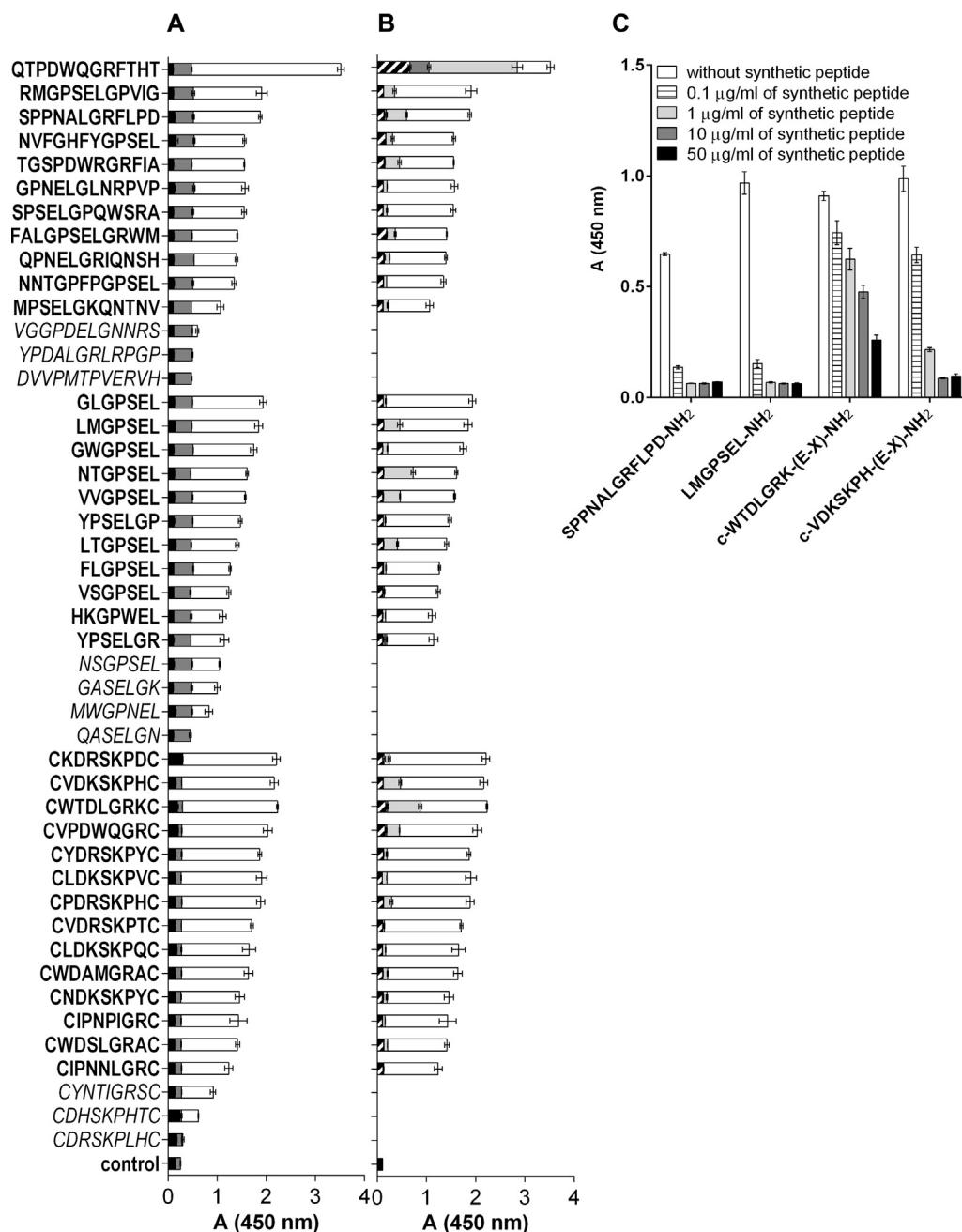


FIG E1. Characterization of peptides obtained by biopanning of phage display libraries with anti-Ap1 m 1 IgG. **A**, Binding of peptides displayed on phage to the immobilized target antibodies (white bars), control antiserum (gray bars), and background (black bars). **B**, Displacement of peptides displayed on phage from target antibodies with 1, 5, and 10 $\mu\text{g/mL}$ of nAp1 m 1 (light gray, dark gray, and striped bars, respectively). The peptides marked in italics showed low signals and were not tested in competitive ELISA. Wild-type phage without displayed peptide served as negative control. **C**, Competition of peptides displayed on phage with the corresponding synthetic analogs for binding to target antibodies demonstrates that the synthetic mimotopes adopt the same conformation in solution as displayed on phage. White bars represent binding of the peptides displayed on phage to target antibodies with no synthetic peptide added. Striped, light gray, dark gray, and black bars represent the binding signal after preincubation of the same peptides displayed on phage with increasing concentration of corresponding synthetic analogs. The data are presented as the mean $\pm$ SD of 3 individual experiments.

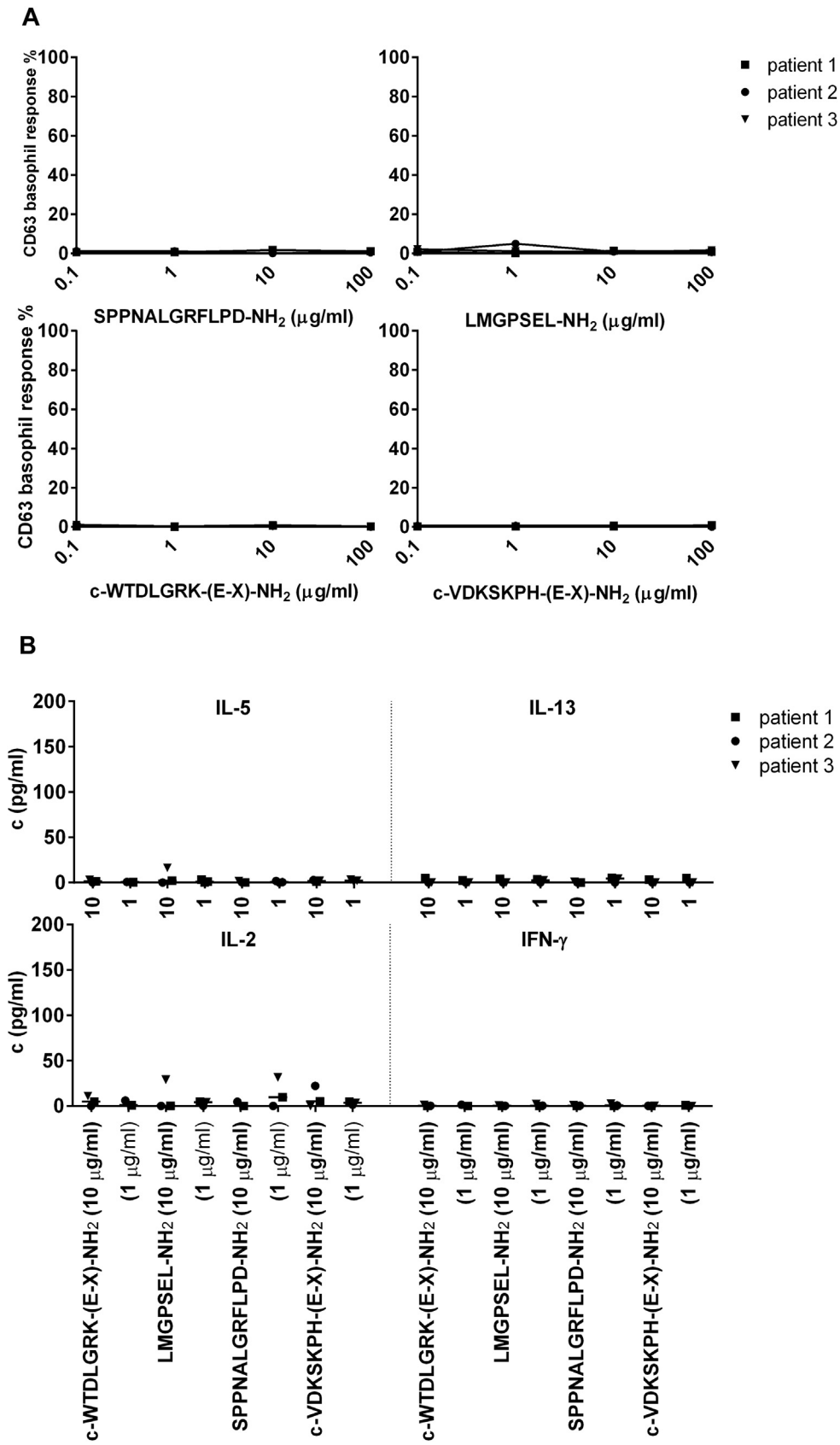


FIG E2. A, Basophil response determined by the percentage of CD63-expressing cells in bee venom–allergic patients (patients 1-3) after addition of synthetic peptide mimotopes. B, Cytokines production after 48-hour stimulation of PBMCs in bee venom–allergic patients (patients 1-3) with synthetic peptide mimotopes. The middle lines represent median.

TABLE E1. Characteristics of honeybee venom–allergic patients

Patient	Sex	Age at diagnosis (y)	sIgE* bee venom (kU/L)	sIgE* Api m 1 (kU/L)
Patient 1	F	73	59.3	11.9
Patient 2	F	33	30.5	16.1
Patient 3	F	35	2.0	0.471
Patient 4	M	28	0.97	1.3
Patient 5	M	47	0.55	0.67
Patient 6	F	72	2.10	1.16
Patient 7	M	55	6.94	0.55
Patient 8	F	49	2.47	2.38
Patient 9	M	48	9.71	13.9
Patient 10	F	48	33.5	19.6
Patient 11	M	31	4.68	1.5
Patient 12	M	35	2.91	0.66

F, Female; M, male; sIgE, specific IgE.

*sIgE levels were measured by using CLIA Immulite (Siemens, Erlangen, Germany). Serum samples were collected at the University Clinic of Respiratory and Allergic Diseases, Golnik, Slovenia. The patients were included in the study before the introduction of bee venom immunotherapy. All 12 patients had positive sIgE to Api m 1 and were used to test IgE reactivity of peptides. Patients 1, 2, and 3 with Mueller grade III (patient 3) or grade IV (patients 1 and 2) anaphylactic reactions to bee sting were selected for evaluation of the allergenic activity and T-cell response-modulating potential of peptide mimotopes. The study was approved by National Medical Ethics Committee of Republic of Slovenia (No. 35/06/14). All patients willingly donated their blood for research.