

Identification and characterization of major cat allergen Fel d 1 mimotopes on filamentous phage carriers

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ABSTRACT

Cat allergy is one of the most prevalent allergies worldwide and can lead to the development of rhinitis and asthma. Thus far, only allergen extracts from natural sources have been used for allergen-specific immunotherapy. However, extracts and whole allergens in immunotherapy present an anaphylaxis risk. Identification of allergen epitopes or mimotopes has an important role in development of safe and effective allergen-specific immunotherapy. Moreover, with a suitable immunogenic carrier, the absence of sufficient immune response elicited by short peptides could be surmounted. In this study, we identified five structural mimotopes of the major cat allergen Fel d 1 by immunoscreening with random peptide phage libraries. The mimotopes were computationally mapped to the allergen surface, and their IgE reactivity was confirmed using sera from cat-allergic patients. Importantly, the mimotopes showed no basophil activation of the corresponding cat-allergic patients, which makes them good candidates for the development of hypoallergenic vaccine. As bacteriophage particles are becoming increasingly recognized as immunogenic carriers, we constructed bacteriophage particles displaying multiple copies of each selected mimotope on major phage coat protein. These constructed phages elicited T cell-mediated immune response, which was predominated by the type 1 T cell response. Mimotopes alone contributed to the type 1 T cell response by promoting IL-2 production. Fel d 1 mimotopes, as well as their filamentous phage immunogenic carriers, represent promising candidates in the development of hypoallergenic vaccine against cat allergy.

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1. Introduction

Allergic diseases have been increasing over the last several decades, and cat allergy is no exception. It is considered to be one of the major risk factors for the development of rhinitis (Perzanowski et al., 2002), which is an IgE-mediated allergic disease that often progresses to asthma (Becker et al., 2013). The major cat allergen Fel d 1 is a secretoglobulin that is secreted by the cat's sebaceous glands and squamous epithelial cells (Ohman et al., 1974). IgE epitopes of Fel d 1 play an important role in the IgE-mediated allergic response. Therefore, identification of epitopes or mimotopes, peptide mimics of the epitope's physicochemical properties (Pali-Scholl and Jensen-Jarolim, 2008), could aid in the development of allergen-specific immunotherapy (ASIT). After a century, ASIT remains the only etiologic treatment for allergic disorders that

can alter the natural course of the disease (Viswanathan and Busse, 2012; Abramson et al., 2010) and not just ease allergic symptoms. The main goal for ASIT is the development of a safe therapeutic construct with maintained or heightened immunogenicity in parallel with reduced allergenic activity (Broide, 2009; Valenta et al., 2010; Focke et al., 2010). However, when using whole unaltered allergens, natural or recombinant, there is always a risk of anaphylaxis, which can lead to death. For this reason, short peptides representing epitopes or mimotopes of an allergen are getting their opportunity to improve ASIT. The main goal of such therapeutics is to reduce binding to IgE on mast cells or basophils and therefore anaphylaxis, while targeting allergen-specific T cells (Valenta et al., 2010). Small peptides usually do not elicit a sufficient immune response when injected on their own; however, by using a suitable immunogenic carrier, this could be surmounted. Bacteriophage particles have garnered recognition in the field of molecular allergology due to their value not only in competitive immunoscreening (Yang et al., 2015; Christiansen et al., 2015) but also as immunogenic carriers of allergen mimotopes (Scholl et al., 2002). They integrate epitope

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discovery technology and immunization functions into a single platform. In this study, we first used random peptide phage libraries to identify and characterize structural mimotopes of the major cat allergen Fel d 1. Subsequently, we constructed filamentous bacteriophages as immunogenic carriers displaying multiple copies of individual mimotope. We evaluated the allergic immune response and immunogenic properties of the carrier and mimotopes.

2. Materials and methods

2.1. Patients' blood

Blood from thirteen individuals was collected. Seven patients (patient 1–7) were diagnosed with cat allergy, two (atopic controls 8 and 9) were not allergic to cats but had other allergies, and four were healthy individuals without any reported allergies (healthy controls 10–13) (Table 1). This study was approved by the National Medical Ethics Committee of Republic of Slovenia (No. 35/06/14). All patients willingly donated their blood for research.

2.2. Purification of rabbit antiserum specific to Fel d 1

Rabbit IgG was affinity-purified from anti-Fel d 1 rabbit antiserum (INDOOR Biotechnologies, Charlottesville, Virginia, USA) using a recombinant Fel d 1 allergen (rFel d 1; INDOOR Biotechnologies), which was covalently bound to Dynabeads M-280 Tosylactivated (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions.

2.3. Binding of purified rabbit IgG to rFel d 1

To confirm binding of the purified rabbit IgG to rFel d 1, Maxisorp microtiter plates (Thermo Fisher Scientific) were coated with 2 µg/mL of rFel d 1 in PBS overnight at 4 °C and incubated with 5% skim milk in PBS for 2 h at room temperature. A washing buffer of 0.5% Tween 20 in PBS (PBST) was used. Next, the plates were incubated with purified rabbit IgG for 1 h at room temperature. For detection, the goat anti-rabbit IgG conjugated with horse radish peroxidase (HRP) (Thermo Fisher Scientific) diluted to 1:5000 was added to the wells and incubated for 1 h at room temperature. TMB Super Tracker (ImmunoO₄, Westminster, UK) with added 0.006% H₂O₂ was used as a substrate. The reaction was terminated with 2 M H₂SO₄, and the absorbance was measured at 450 nm with the Tecan Safire microtiter plate reader (Tecan Group AG, Männedorf, Switzerland).

2.4. Immunoscreening

Affinity-purified rabbit IgG specific to rFel d 1 was immobilized onto protein G or protein A coupled to Dynabeads (Thermo Fisher Scientific), incubated in 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, Missouri, USA) in PBST for 30 min and used as a target in biopanning. Dodecamer, heptamer and cyclic heptamer random peptide phage libraries (New England Biolabs, Ipswich, Massachusetts, USA) were screened according to the manufacturer's instructions. Briefly, 10¹¹ plaque forming units (pfu) displaying 10⁹ random peptides were incubated with 15 µL of immobilized Dynabeads for 1 h at room temperature. Unbound phages were washed with PBST. Bound phages were eluted with either rFel d 1 or with 0.1 M glycine buffer with pH 2.2 and amplified in the *Escherichia coli* strain ER2738 to be used in the next rounds of biopanning. After the third round, *E. coli* were infected with the eluted phages and grown on LB plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl β-D-1-thiogalactopyranoside (IPTG). The plates were incubated overnight at 37 °C. Individual plaques (20–30) were picked for each

type of elution and amplified. Individual phage clones were purified from the supernatant using PEG/NaCl precipitation. DNA from the phage clones showing binding in an ELISA were isolated and sequenced to determine the displayed peptide primary structure.

2.5. Binding of phage clones to anti-Fel d 1 rabbit antiserum

The wells of the microtiter plates were coated overnight at 4 °C with anti-Fel d 1 rabbit antiserum diluted to 1:1000 in 0.1 M NaHCO₃ (pH 8.6). To detect and eliminate possible binders to antibodies constant regions control rabbit antiserum raised against procathepsin X (Biogenes, Berlin, Germany) was coated at same dilution and in the same buffer to microtiter plate wells. The wells were blocked with 5% skim milk in PBS and washed with PBST. Next, the supernatants (100 µL) with individual amplified phage clones were added to the wells and incubated for 1 h at room temperature. For detection, mice anti-M13 monoclonal antibodies conjugated with HRP (GE Healthcare, Little Chalfont, UK) diluted to 1:5000 were incubated for 1 h at room temperature and the TMB Super Tracker (ImmunoO₄) with added 0.006% H₂O₂ was used as a substrate. The reaction was terminated with 2 M H₂SO₄, and the absorbance was measured at 450 nm with a microtiter plate reader. The ratio of the ELISA signal of the test phage wells to the wild-type phage (control phage) wells was calculated.

2.6. Competition ELISA

The microtiter plate wells were coated, blocked and washed as described above. Next, selected, purified phage clones (10⁹ pfu/well) were added to the wells and allowed to compete for the target antibody binding site with rFel d 1 (0.25 µg/well). After 1 h of incubation at room temperature, anti-M13 monoclonal antibodies conjugated with HRP (GE Healthcare) diluted to 1:5000 were added. Detection was performed as above.

2.7. SDS-PAGE and western blot

SDS-PAGE was performed according to the Laemmli protocols (Laemmli, 1970). Loading dye and dithiothreitol was added to selected phage clones (from phage libraries) diluted in PBS. The mixture was boiled for 5 min, and 35 µL containing 10¹² phage particles were pipetted into the wells of a 15% polyacrylamide gel. Phage titers were determined by microbiological titration. Electrophoresis was performed in a vertical slab gel apparatus (Bio-Rad, Hercules, California, USA) for 2–2.5 h at 100 V. The proteins were blotted onto a 0.45 µm pore nitrocellulose membrane (GE Healthcare) for 1.5–2 h at 100 V in a wet electroblotting system (Bio-Rad).

For immunodetection, the membrane was blocked with 5% skim milk in Tris-buffered saline/0.05% Tween 20 (TBST) for 3 h at room temperature. Next, the membrane was incubated with human serum diluted to 1:1 with TBST or with Fel d 1 rabbit antiserum diluted to 1:1000 with TBST overnight at 4 °C. After washing the membrane 3 times with TBST, goat anti-human IgE antibodies conjugated with HRP diluted to 1:2000 with TBST or diluted goat anti-rabbit IgG conjugated with HRP were incubated with the membrane for 2 h at room temperature. The SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) was used for detecting the HRP signal.

2.8. Mapping of peptides to the surface of Fel d 1

Selected peptides were mapped on the Fel d 1 surface using The Peptide Server (<http://pepitope.tau.ac.il/>). The epitope mapping algorithm Pepsurf, Mapitope or a combination of them was used for mapping peptides onto the Fel d 1 surface. A file from the RCSB Protein data bank with PDB ID 1ZKR was used as a target molecule. The

surface locations of the amino acids that could represent potential epitopes were presented using ViewerLite.

2.9. Construction of filamentous phage mimotope carriers (FPMC)

Filamentous virion particles displaying approximately 150 copies (Kouzmitcheva et al., 2001) of mimotopes fused to the major coat protein pVIII were constructed as previously described (<http://www.biosci.missouri.edu/smithGP/>). The oligonucleotides encoding individual mimotopes were prepared by Sigma-Aldrich, and the individual oligonucleotide pairs were hybridized to form double stranded inserts flanked by HindIII and PstI sites at the 5' and 3' ends of the coding strands, respectively. The phage vector f88-4 (GenBank accession number AF218363) (Kouzmitcheva et al., 2001) was obtained from Prof. G. P. Smith's laboratory (Department of Biology, University of Missouri, Columbia, MO, USA). Double-stranded f88-4 DNA was isolated using the GenElute HP Plasmid Miniprep Kit (Sigma-Aldrich) and digested with HindIII and PstI restriction enzymes. A double-cut/cleaved vector was isolated by agarose gel electrophoresis and a QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). The individual inserts were ligated (T4 DNA ligase, New England BioLabs) into a linearized f88-4 vector followed by heat-shock transformation of competent *E. coli* strain TG1 cells using individual ligation mixtures. Colonies harboring recombinant vectors with correct inserts were screened using colony-polymerase chain reaction and agarose gel electrophoresis. Correctly prepared constructs were confirmed by DNA-sequencing. The FPMC particles were amplified, isolated and purified from *E. coli* TG1 supernatant using PEG/NaCl precipitation.

2.10. Comparison of anti-Fel d 1 IgE in patients' sera

The microtiter wells were coated with 2 µg/mL of rFel d 1 in PBS overnight at 4 °C. Blocking and washing were performed as described above. Human sera from patients 5–7 and healthy control 11 diluted to 1:10 were added to the wells for 1.5 h at room temperature. After washing, goat anti-human IgE antibodies conjugated with HRP (Thermo Fisher Scientific) diluted to 1:2000 were incubated for 1 h at the room temperature. Further detection steps were performed as described above. Each assay was performed in triplicates. Mean values and standard deviations are represented in Fig. 4A. The serum producing the highest signal in ELISA to rFel d 1 was used to compare binding of peptide mimotopes on FPMCs and selected library phage clones to human IgE. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc analysis.

2.11. Comparison of FPMC with library phage clones

The microtiter plate wells were coated with 10^{11} pfu in PBS of FPMCs displaying individual peptide mimotopes fused to major coat protein pVIII and library phage clones displaying the same peptide on the minor coat protein pIII overnight at 4 °C. The wells were blocked and washed as in the ELISA assays described above. The displayed peptide mimotopes were detected with anti-Fel d 1 rabbit antiserum diluted to 1:1000 for 1 h at room temperature, and goat anti-rabbit IgG conjugated with HRP diluted to 1:5000 was added for 1 h at room temperature. Further detection steps were performed as described above. For each library phage and FPMC and for control phage assay was performed in triplicates. Mean values and standard deviations are represented in Fig. 4B. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-hoc analysis.

2.11.1. Binding of FPMCs and library phage clones to human IgE

The microtiter wells were coated overnight at 4 °C with rabbit anti-fd bacteriophage antibodies (Sigma-Aldrich) in 0.1 M NaHCO₃. Constructed FPMC were diluted in PBS, added to the wells (10^{10} pfu/well) and incubated for 2 h at room temperature. Blocking and washing were performed as described above. Human serum from patient 5 and healthy control 11 diluted 1:10 was added to the wells and incubated for 1.5 h at room temperature. After washing, goat anti-human IgE antibodies conjugated with HRP (Thermo Fisher Scientific) diluted to 1:2000 were incubated for 1 h at room temperature. Further detection steps were performed as described for ELISA above. Each assay was performed in triplicates. Mean values and standard deviations are represented in Fig. 4C. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-hoc analysis.

2.11.2. Basophil activation test

Basophil activation tests were performed as described previously (Lalek et al., 2010; Kosnik et al., 2005). Briefly, whole-blood samples (patients 1–3, atopic controls 8 and 9, and healthy control 12) were incubated with various concentrations of constructed FPMCs (final concentrations from 1×10^{12} to 1×10^8 pfu/mL) or rFel d 1 (final concentrations from 333.3 to 0.0082 ng/mL) and positive controls, 0.55 µg/mL of anti-FcεRI mAb (Bühlmann Laboratories AG, Schönenbuch, Switzerland) and 2 µM fMLP (Sigma-Aldrich) at 37 °C for 15 min. Degranulation was stopped by chilling on ice, after which FITC-conjugated anti-CD63 mAb, PE-conjugated anti-CD123 mAb, and PerCP-conjugated anti-HLA-DR mAb (BD Biosciences, San Jose, California, USA) were added and incubated for 20 min on ice. Finally, whole blood probes were lysed, washed, fixed, and analyzed within 2 h on a FACSCalibur flow cytometer (BD Biosciences). The data were acquired using an FL2 threshold that eliminated most CD123-negative cells, and at least 600CD123-positive cells were acquired per sample. Basophils were identified as low side scatter, CD123-positive, and HLA-DR-negative cells, and the quantitative percentage determination of degranulated basophils was measured in FL1. The threshold value for diagnostically positive results was defined as 15% of CD63-positive basophils. This threshold was described previously (Kosnik et al., 2005; Korosec et al., 2009).

2.11.3. Cytokine measurements

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole-blood samples from three cat-allergic patients (patients 1–3), one patient with other allergies (atopic control 9) and two healthy individuals (healthy controls 12 and 13). Briefly, heparinized whole blood was carefully added to the same volume of Ficoll Paque (GE Healthcare) and centrifuged at 1800 rpm for 30 min at room temperature. The plasma was pipetted away, and the middle layer (containing PBMCs) was carefully transferred to a new tube. The cells were washed using RPMI-1640 medium (Thermo Fisher Scientific) and centrifuged at 1600 rpm for 5 min. The supernatant was removed, and the cells were washed with the same medium and centrifuged at 1600 rpm for 5 min. The supernatant was removed again, and the cells were resuspended and counted. PBMCs were cultured in RPMI-1640 Medium HEPES Modification (Sigma-Aldrich), fetal bovine serum (Sigma-Aldrich) and 200 mM L-glutamine (Sigma-Aldrich). Constructed FPMCs were used at a final concentration of 1×10^{12} pfu per sample and incubated for 48 h. Four cytokines, namely IL-2, IL-5, IL-13 and IFN-γ, were quantified in the supernatants with the Cytometric Bead Array (CBA; BD Biosciences) according to the manufacturer's protocol.

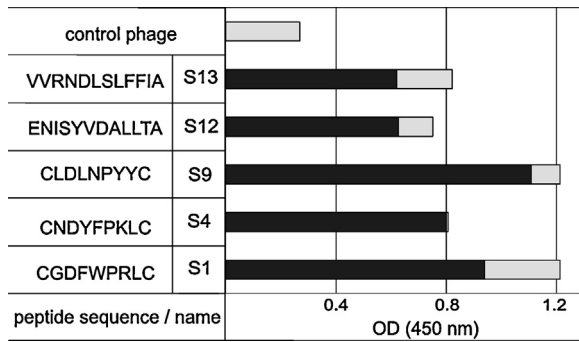


Fig. 1. Peptides (sequences and names) with highest avidity and their ability to compete with allergen rFel d 1. ELISA signals obtained after incubation of immobilized target antibodies with selected peptide displaying phages and control wild-type phage with (black bars) or without (grey bars) addition of competitive ligand rFel d 1. Background (signal obtained on BSA coated wells) for each peptide displaying phage and control phage was subtracted.

3. Results and discussion

3.1. Selection of mimotopes from phage peptide libraries

Two selections, using a specific or non-specific elution, with each phage library were performed. Fel d 1 rabbit antiserum was affinity-purified and binding of purified rabbit IgG to recombinant Fel d 1 was confirmed with ELISA (data not shown) to ascertain the appropriate paratope characteristics of the target. We presumed that the most important Fel d 1 epitopes are mutual for rabbit IgG as well as patients' IgE, which was later confirmed with selected mimotopes. After three rounds of biopanning, approximately 50 individual colonies from each selection were selected, amplified and tested by ELISA. Phage clones that revealed an ELISA absorbance higher than 0.5 and ELISA signal ratio in target-coated and BSA-coated wells higher than 2 were sequenced and further evaluated. ELISA signals for five best binders and control phage are given in Fig. 1. Displacement of selected phage from the target by the allergen was also determined (Fig. 1). Two of selected displayed peptides were dodecamers and three were cyclic heptamers. All were selected with specific elution conditions using recombinant Fel d 1. None of the linear heptapeptides exhibited sufficient binding towards the target paratopes. They showed binding to control rabbit antiserum, which is a characteristic of target-unrelated peptides directed towards antibody constant regions (Vodnik et al., 2011).

Peptide displacement from the target antibody by allergens indicates the mimotope nature of the peptide. Competition ELISA

revealed that phages displaying peptides S1 and S13 (phage S1 and phage S13) had the highest displacement percentage, approximately 20%. Phages displaying peptide S12 (phage S12) had approximately 15% displacement, and phages displaying peptide S9 (phage S9) exhibited approximately 7% displacement. Displacement of phage S4 from the target was very insignificant, however sufficient to allow for enrichment with specific elution conditions. The peptide and allergen epitope might have different binding sites on the antibody, especially when non-specific elution is utilized in the biopanning procedure. Non-specific elution may also enrich peptides with higher affinity towards target antibody paratopes compared to allergen epitopes. Nonetheless phage clones with highest ELISA target to background ratio were obtained with specific elution. Since phage S4 did not bind to control rabbit antiserum and was structurally similar to S1 we hypothesized that peptide S4 mimics an allergen epitope and included it in further experiments.

3.2. Binding of peptide mimotopes to IgE in allergic patients' sera

We confirmed the ELISA results presented in Fig. 1 via detection of membrane-bound pIII-peptide fusion proteins with rabbit antiserum. Both wild-type and peptide fusion pIII ran on gel with an apparent molecular weight of 60–65 kDa (Fig. 2A). Selected peptides expressed as fusions with phage coat protein pIII bound anti-Fel d 1 IgG from rabbit antiserum used in panning protocols, whereas pIII from wild-type phage did not exhibit this binding (Fig. 2B, first row). To establish the mimotope characteristics of selected peptides, we further tested the binding of peptide-pIII fusion proteins to IgE from cat-allergic patients' sera. All selected peptides fused to pIII were able to bind IgE from cat-allergic patients (patient 1–4), in contrast to wild-type pIII, which had poor non-specific binding or no IgE reactivity (Fig. 2B). The observed non-specific reactivity may arise from protein pIII structural similarities with some other protein/allergen recognized by IgE, which might be expected for patients 1–4, who have other allergies.

Compared to signals obtained with IgG from rabbit antiserum, signals obtained with IgE from patients' sera were less intense due to lower IgE concentrations. Cat allergy is associated with lower IgE titers to allergen compared to other allergies (Erwin et al., 2007, 2009). For example, patient 2, despite marked symptoms of cat allergy, had IgE concentration below the threshold 0.35 kU/L, comparable to healthy controls. However, patient 1 serum harbored the highest IgE concentration (Table 1) and produced the strongest and most clear signal without unspecific binding and confirmed that selected peptides were mimotopes of major cat allergen Fel d 1 (Fig. 2A). Some IgE reactivity also appeared in the serum from healthy control 10. Even non-allergic patients have

Table 1
Patients' characteristics.

	sIgE* cat (kU/L)	Basophil activation		Cat allergy symptoms	Other allergies
		cat epithelium	rFel d 1		
Patient 1	56.5	yes	yes	yes	yes
Patient 2	<0.35	no	yes	yes	yes
Patient 3	1.21	yes	yes	yes	yes
Patient 4	8.47	not tested	not tested	yes	yes
Patient 5	29.3	not tested	not tested	yes	no
Patient 6	7.41	not tested	not tested	yes	yes
Patient 7	1.89	not tested	not tested	yes	no
Atopic control 8	0.40	no	no	no	yes
Atopic control 9	<0.35	no	no	no	yes
Healthy control 10	<0.35	not tested	not tested	no	no
Healthy control 11	<0.35	not tested	not tested	no	no
Healthy control 12	<0.35	no	no	no	no
Healthy control 13	<0.35	not tested	not tested	no	no

sIgE*— specific immunoglobulin E were measured by using FEIA ImmunoCAP (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

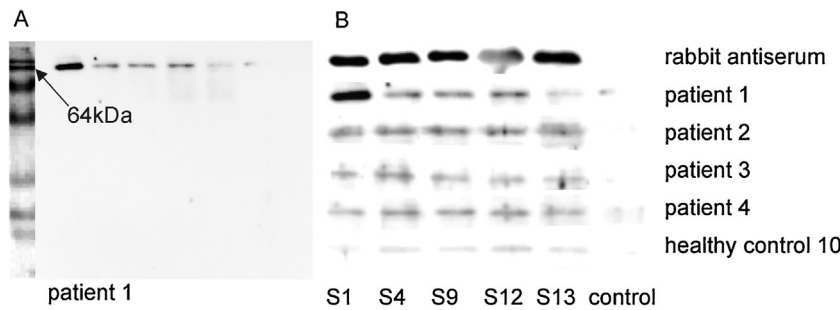


Fig. 2. Binding to peptide mimotopes on minor coat protein pIII determined by SDS-PAGE and western blot. (A) Binding of IgE from patient 1. (B) Binding of IgE from cat-allergic patients 1–4 and healthy control 10 and binding of rabbit antiserum IgG. Protein pIII from wild-type phage was used as control.

exhibited low amounts of IgE against antigens/allergens (Carosso et al., 2007; Wittig et al., 1980). Cross-reactivity among allergens is well-documented (Bartra et al., 2009; Vieths et al., 2002; Popescu, 2015), and selected mimotopes might represent more universal allergen epitope patterns not specific merely for Fel d 1 but capable of binding related paratopes on a fraction of IgE even in non-allergic individuals.

3.3. Computational alignment of peptides on the allergen

Mimotopes were aligned on the surface of the Fel d 1 protein, which contains two chains: A and B. Peptides S12 and S13 exhibited homology to parts of both chains (Fig. 3A). Peptide S12 aligned with E₃₆N₃₇L₃₅A₂₆Y₂₈T₁₃₆K₂₉A₃₀L₃₁L₁₃₂T₁₃₅G₁₃₁ on chain A and with N₉₁N₈₉I₁₂₅S₁₂₆V₁₂₈D₁₃₀G₁₃₁L₁₂₉L₁₃₂T₁₃₆S₁₃₉ on chain B (Fig. 3A, yellow). Peptide S13 aligned with V₁₂₀I₁₂₅R₁₂₇N₃₇E₃₆L₃₅L₃₁L₁₃₂T₁₃₆Y₂₈A₂₆ on chain A and with V₁₂₈I₁₂₅N₈₉D₁₃₀L₁₂₉L₁₃₂L₃₁V₃₃L₃₅A₃₀ on chain B (Fig. 3A, red). Peptides S1, S4 and S9 aligned with chain B. Peptide S1 aligned with G₁₃₁D₁₃₀F₈₅Y₈₁P₇₈L₆₉ (Fig. 3B, blue), peptide S4 with N₈₉D₁₃₀F₈₅Y₈₁P₇₈E₇₅ (Fig. 3B, yellow) and peptide S9 aligned with L₁₂D₁₁L₅₈N₅₆P₁₀₇ (Fig. 3B, red).

The predicted epitopes of S12 and S13 exhibited overlap with both chains (Fig. 3A), with eight shared amino acids (26, 28, 31, 35, 36, 37, 132 and 136) on chain A and six (89, 125, 128, 129, 130 and 132) on chain B (orange). Cys constrained peptides S1 and S4 share the strongest primary structure similarity among the selected peptides. The predicted epitopes matched to these two cyclic peptides also exhibited common amino acids (78, 81, 85 and 130) on chain B (green). We speculate that these overlapping areas that contain four to eight amino acids comprise antigen-antibody interaction site. The amino acid at position 89 on chain B was mapped by three peptides (S4, S12 and S13), and the amino acid at position 130 was mapped by four (S1, S4, S12 and S13). This suggests that the main epitope could be located on chain B.

3.4. Construction and characterization of FPMCs displaying mimotopes on the pVIII coat protein

Library phage particles display 1–5 copies of peptide on the pIII minor coat protein. To obtain a higher peptide copy number on the phage coat, we constructed recombinant FPMCs displaying selected mimotopes on the pVIII major coat protein as described by Kouzmitcheva et al. (2001). The filamentous phage consists of approximately 2700 copies of the major coat protein pVIII. Under fully induced conditions, approximately 150 copies of peptide fused to pVIII are expressed on the phage surface (Kouzmitcheva et al., 2001). Due to increased avidity to rabbit antiserum, constructed FPMCs exhibited 1.5–3.5-fold higher signals in ELISA than library phages (Fig. 4, B), which confirmed the higher copy number of displayed mimotopes. Conversely, the avidity to patients' IgE was

far less pronounced (Fig. 4C). Cat-allergic patients harbor low and variable IgE titers, and their paratope repertoire is unique (Erwin et al., 2007, 2009). Serum from patient 5 demonstrated the highest binding signal to rFel d 1 in ELISA (Fig. 4A) and had a markedly elevated IgE concentration (Table 1). Therefore, this serum was used to compare binding of peptide mimotopes on FPMCs and the corresponding library phages (Fig. 4C). Only cyclic peptides S1, S4 and S9 displayed higher signals when expressed on FPMC, and the signals were significantly higher only for S1 and S9. Interestingly, the two cyclic mimotopes also demonstrated the highest binding to anti-Fel d 1 rabbit antiserum and the highest ratio of the ELISA signal in test phage wells to wild-type phage (control phage) wells (Fig. 1). We assumed that the sensitivity of the chosen assay was inappropriate to detect significant differences for other, particularly linear, mimotopes. However, using SDS-PAGE and western blotting to compare the signals of gel-separated and membrane-bound mimotopes fused to the proteins pVIII and pIII proved unsuccessful due to the small size of pVIII and stretched spots, even in concentrated gradient gels (data not shown).

3.5. Basophil activation

Basophil activation with rFel d 1 was observed in cat-allergic patients 1–3 but not observed in individuals with other allergies (atopic controls 8 and 9), although the concentration of IgE anti-cat epithelium was slightly elevated in atopic control 8 (Fig. 5A, Table 1).

Responses to control stimuli were as following: anti-FcεRI stimulation was in range from 73.4% to 98.1%, fMLP stimulation was in range from 5.2% to 73.3% and no stimulation was in range from 1.7% to 3.1%. Flow plots with responses to control stimuli and rFel d 1 for patient 1 are shown in Supplementary Fig. 1.

The percentage of basophil activation was determined by subtracting the response produced by control phage from FPMC to determine the amount of basophil activation produced by mimotopes alone (Fig. 5C). Flow plots with responses to bacteriophage particles for patient 1 are shown in Supplementary Figs. 2–4. Some basophil activation was observed upon stimulation with control phages in all subjects (Supplementary Fig. 5). To our knowledge, phage particles have not been used in basophil activation tests yet. Therefore, the mechanism of the activation remains to be unraveled.

By subtracting background activation we demonstrated that mimotopes were not able to activate basophils of cat-allergic patients, of other allergy patients or of non-allergic controls (Fig. 5C). A slight activation was observed when higher phage concentrations were used (above 10^{10} phage particles/mL). The percentage of activation increased with FPMCs concentration, even slightly more in atopic controls 8 and 9 and healthy control 12, who are not allergic to cats (Fig. 5B). The goal of using mimotopes in ASIT is to bind specific IgE (a blocking function) while avoiding IgE

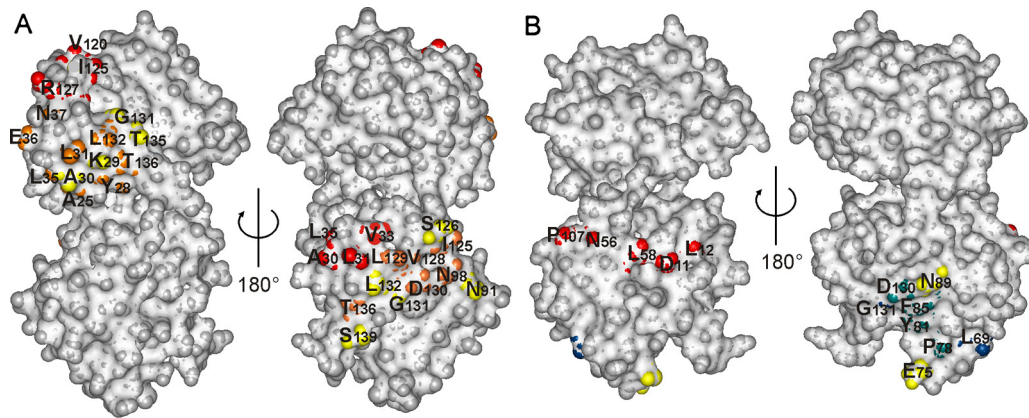


Fig. 3. Alignments of peptides on the surface of Fel d 1 protein. (A) Predicted epitope matched to peptide S12 (yellow) and peptide S13 (red); overlapping aminoacids (orange). (B) Predicted epitope matched to peptide S1 (blue), S4 (yellow) and S9 (red); overlapping aminoacid of peptides S1 and S4 (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

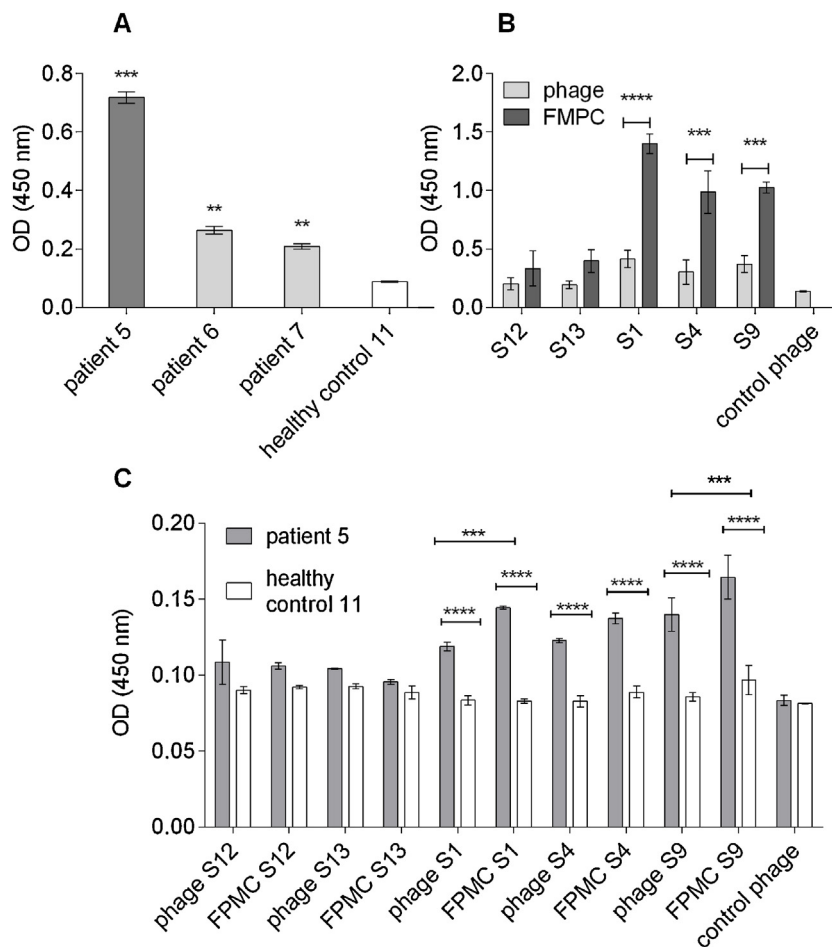


Fig. 4. Comparison of FPMCs and library phages. (A) Binding of IgE from different patient's serum to rFel d 1. Each assay was performed in triplicates. Mean values and standard deviations are represented. Significantly higher affinities compared to healthy control 11 are shown (one-way ANOVA followed by Bonferroni post hoc analysis). (B) Binding of rabbit anti-Fel d 1 IgG to peptide mimotopes, expressed in a low copy number on pIII of library phage clone (phage) and in a high copy number on pVIII on FPMCs. Significantly higher responses between FMPC and library phage with the same mimotope are shown (two-way ANOVA followed by Bonferroni post hoc analysis). (C) Binding of IgE from patient 5 and healthy control 11 to mimotopes expressed in a low copy number (phage) and in a high copy number (FMPC) on phages. Each assay was performed in triplicates. Mean values and standard deviations are represented. Significantly higher responses between FMPC and library phage with the same mimotope and between the same phage for patient 5 and healthy control 11 are shown (two-way ANOVA followed by Bonferroni post hoc analysis). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

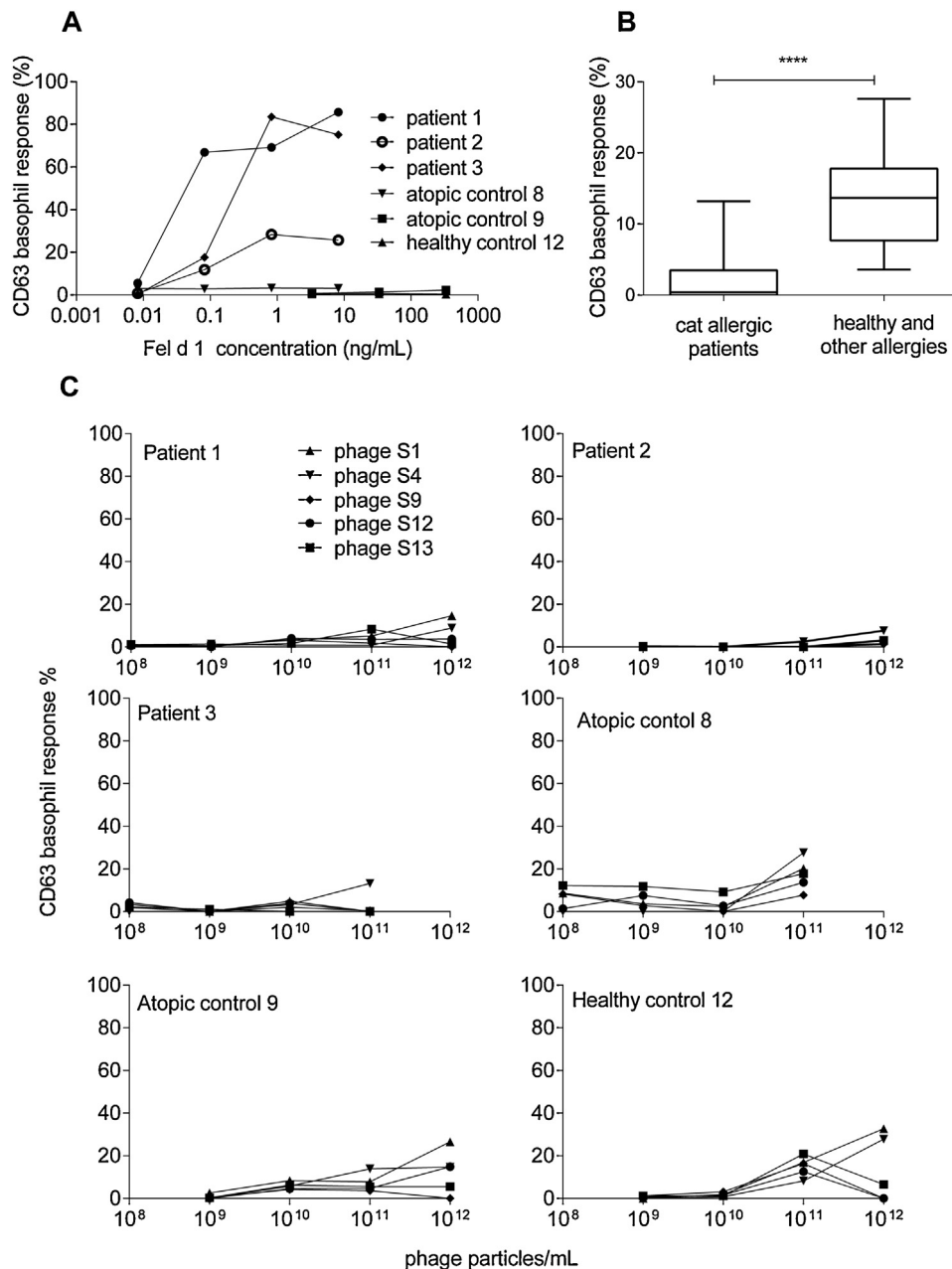


Fig. 5. Basophil response determined by the percentage of surface CD63-expressing cells in cat-allergic patients (patients 1–3), individuals with other allergies (atopic controls 8 and 9) and healthy control 12. (A) Basophil response after stimulation with rFel d 1. (B) Comparison of average basophil response for cat-allergic patients and individuals not allergic to cats. Significantly higher response to mimotopes at 10^{12} FPMCs/mL was shown for individuals not allergic to cats (two-tailed paired *t*-test ($p < 0.0001$)).

cross-linking (an absence of basophil activation), which we demonstrated here.

3.6. Cytokine secretion patterns

Four different cytokines were measured: IL-2 and IFN- γ , which are specific for the Th1 immune response, and IL-5 and IL-13, which are specific for the Th2 immune response. All phage particles, wild-type and FPMCs, elicited T cell-mediated immune response in cat-allergic patients and controls. They activated CD4 $^{+}$ T cells as well as CD8 $^{+}$ T cells. Culturing PBMCs with phages yielded all four measured cytokines. Cytokine IFN- γ was produced at the highest amount, ranging from 150 to 3000 pg/mL. The second most prevalent cytokine was IL-2 with an approximate production of 20 pg/mL

(15–25). The production of IL-13 ranged from 1 to 20 pg/mL. IL-5 exhibited the lowest production at less than 2 pg/mL. These results demonstrate T cell-mediated immune response, especially a type 1 T cell response.

To determine the amount of cytokines produced by mimotopes alone, the cytokine response caused by wild-type phage (Supplementary Fig. 6) was subtracted from the cytokine response caused by FPMCs. We demonstrated that mimotopes alone were responsible only for IL-2 production from T cells (Fig. 6). Cyclic mimotopes (S1, S4 and S9) induced more IL-2 production than linear mimotopes (S12 and S13). We also observed more IL-2 production in cat-allergic patients than in controls. In summary, mimotopes promote IL-2 production, which is specific cytokine for Th1 immune response.

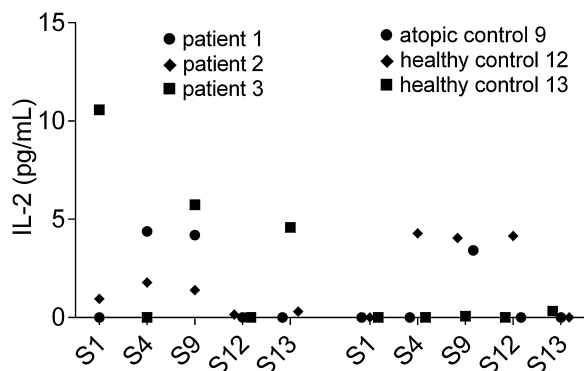


Fig. 6. IL-2 production in cat-allergic patients (left side) and controls (right side) after 48 h stimulation of PBMCs with FPMCs. The amount of produced IL-2 is calculated for effect of mimotopes alone.

4. Conclusion

We identified five peptides through immunoscreening using rabbit anti-Fel d 1 IgG as target antibodies. The peptides displayed the highest binding toward target antibody and four of them competed for the same paratope with recombinant allergen Fel d 1. All five peptides exhibited IgE reactivity to the sera of cat-allergic patients, which confirmed their mimotope nature. Computational mapping on the surface of Fel d 1 suggested that the epitope likely resides on chain B somewhere around aspartic acid at position 130. Because peptides alone cannot elicit immune response, we constructed filamentous bacteriophages as immunogenic carriers displaying multiple copies of an individual mimotope. Filamentous bacteriophages have many advantages for vaccine development. They have simple chemical composition, they are stable and are easy to produce and purify in large amounts. In the context of developing hypoallergenic vaccines their lower immunogenicity due to lower density of presented foreign peptides compared to other immunogenic carriers represents additional advantage. Peptides are protected by the phage carrier, which provides extended degradation time and indicates that phage particles are appropriate vectors for oral and mucosal application (Jensen-Jarolim et al., 1999). Mimotopes were expressed at a high copy number attached to the major coat protein pVIII. This resulted in a higher avidity of FPMCs to target antibodies and IgE from patients' sera. By testing basophil activation, we demonstrated that mimotopes do not have allergenic activity, which makes them good candidates for the development of hypoallergenic vaccines. We also revealed that phage particles are immunogenic carriers that can elicit T cell-mediated immune responses. T cell response was directed preferentially to type 1 T cell response than type 2. As mimotopes promote IL-2 production, they alone also contributed to the type 1 T cell response. Mimotopes, in combination with a suitable immunogenic carrier such as phage particles, can aid in the development of hypoallergenic vaccines for allergen-specific immunotherapy.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2016.02.004>.

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Supplementary Figures

Identification and characterization of major cat allergen Fel d 1 mimotopes on filamentous phage carriers

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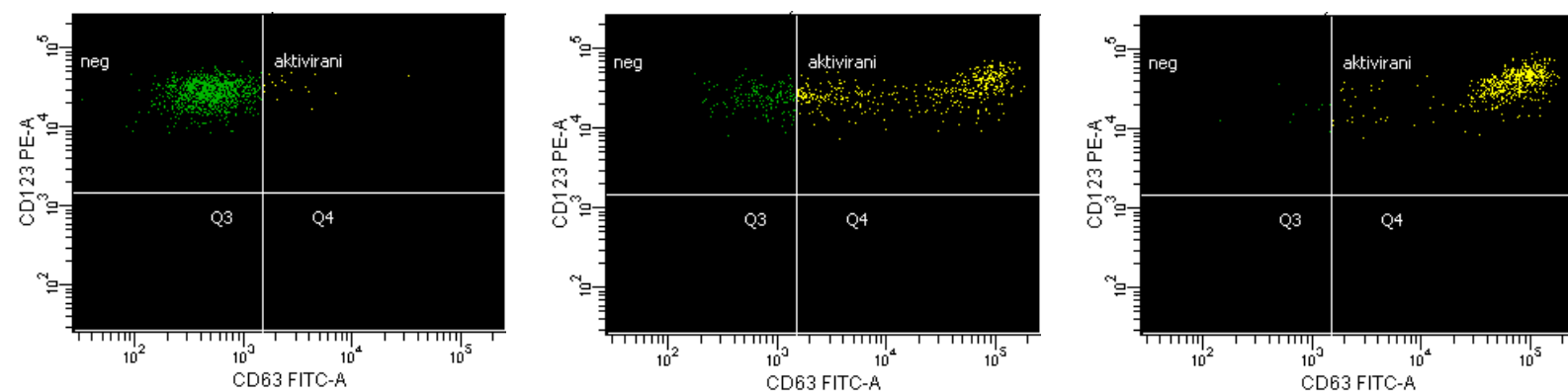
Tel.: +386 1 4769570; E-mail: mojca.lunder@ffa.uni-lj.si

A

no stimulation

fMLP

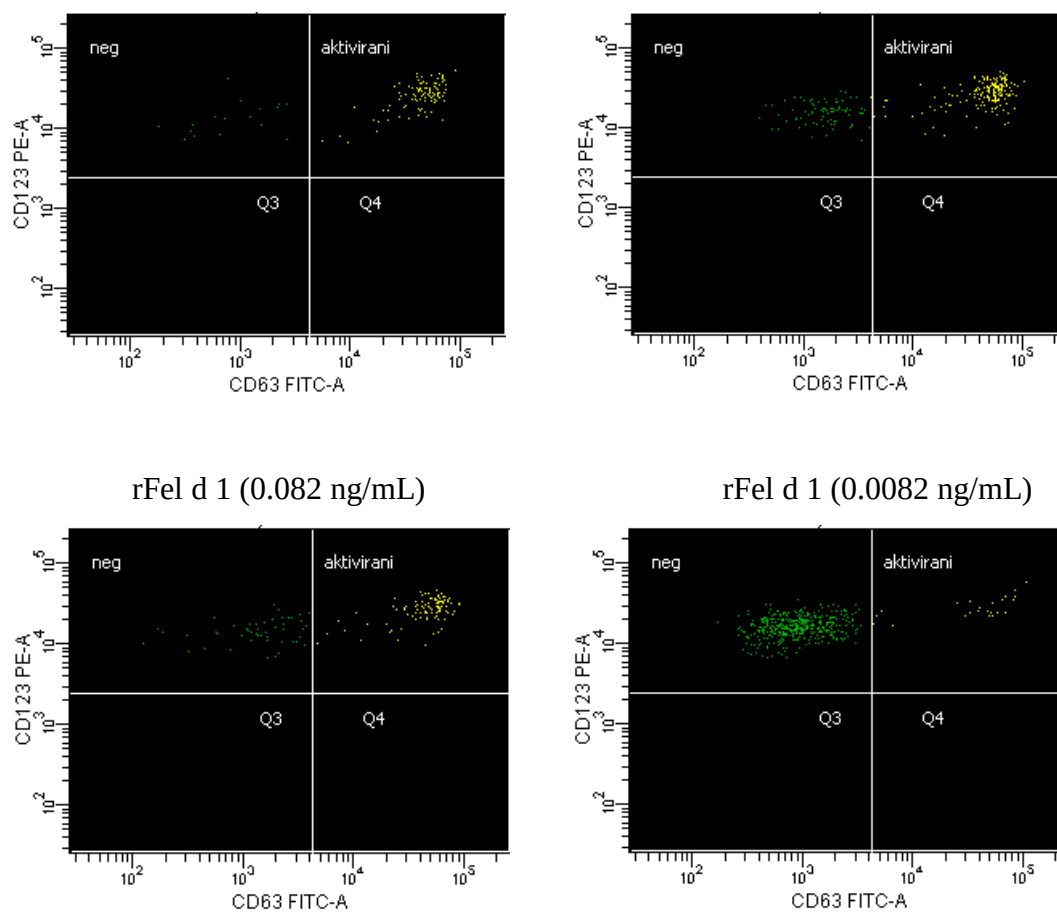
anti-FcεRI



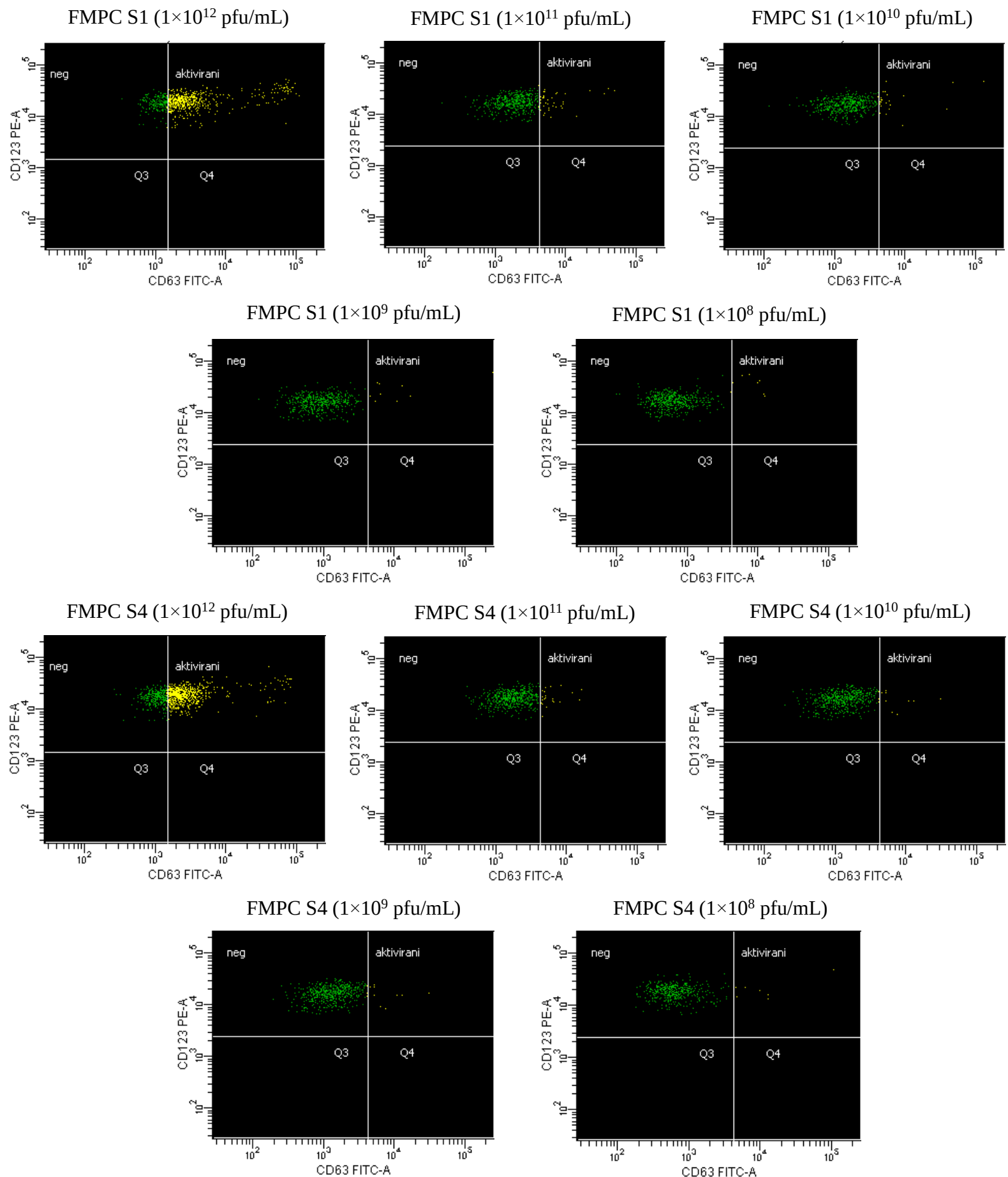
B

rFel d 1 (8.2 ng/mL)

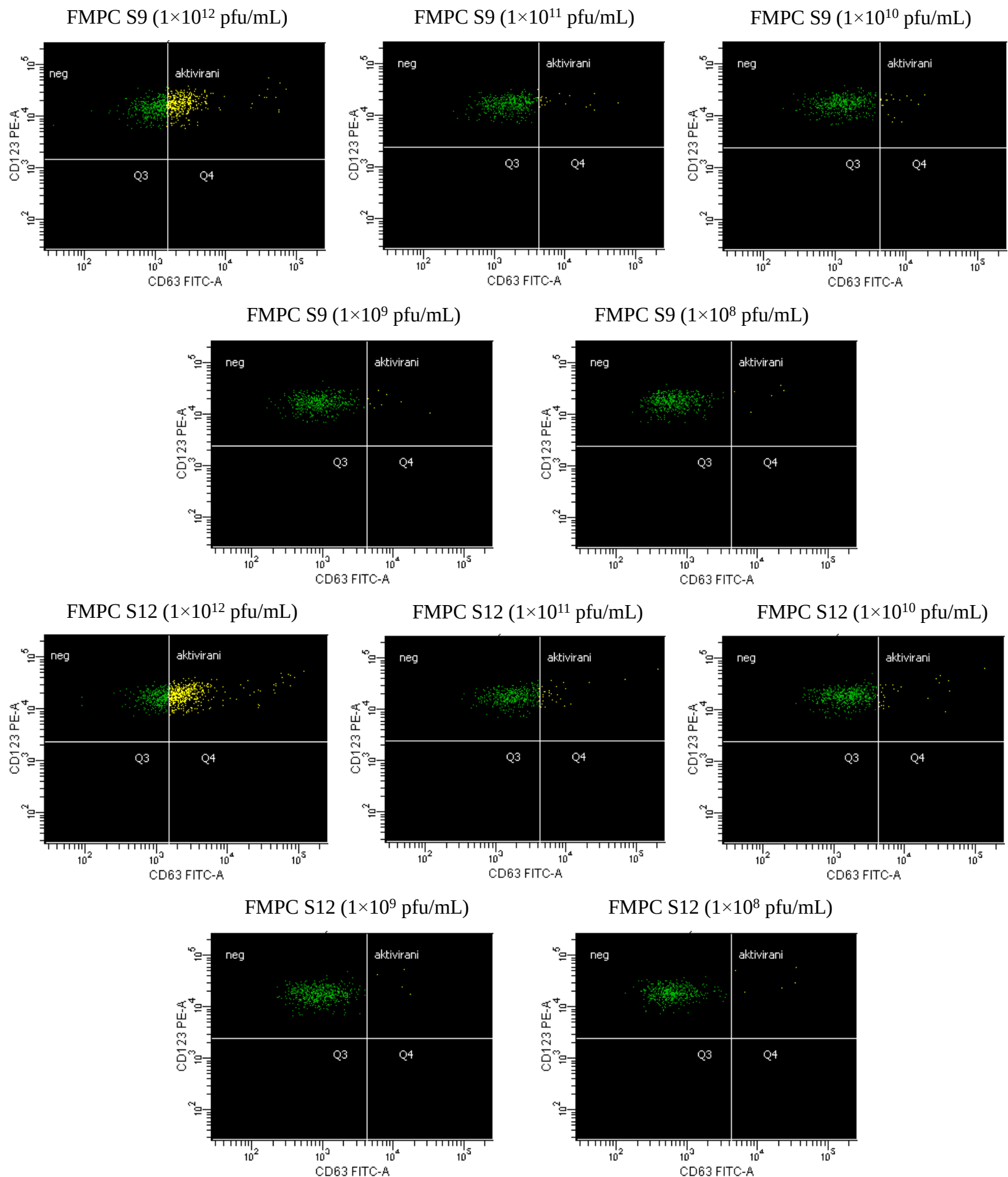
rFel d 1 (0.82 ng/mL)



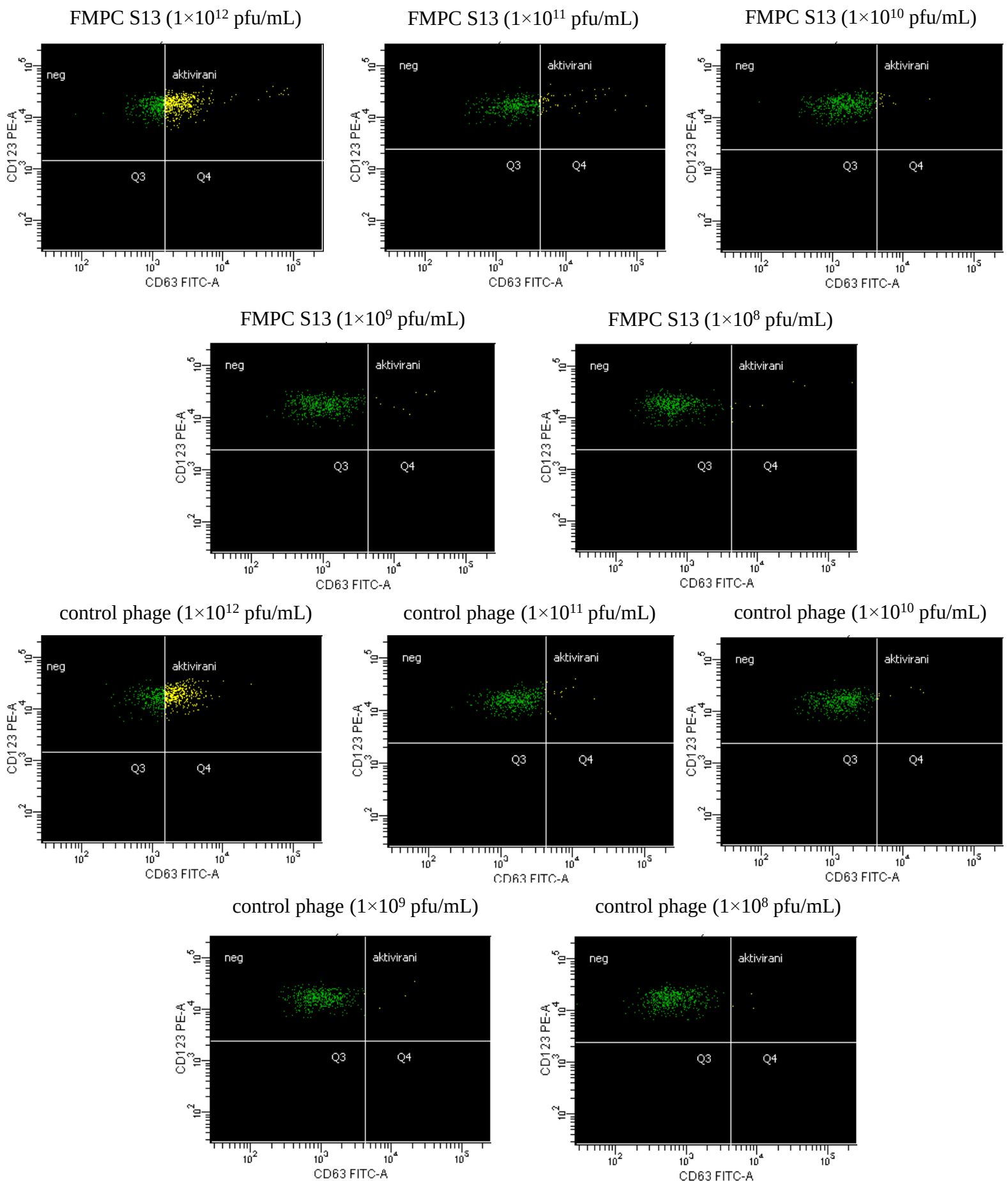
Supplementary Fig. 1. Flow plots of basophil activation tests for patient 1. (A) Control stimuli. (B) Stimulation with different concentrations of recombinant Fel d 1.



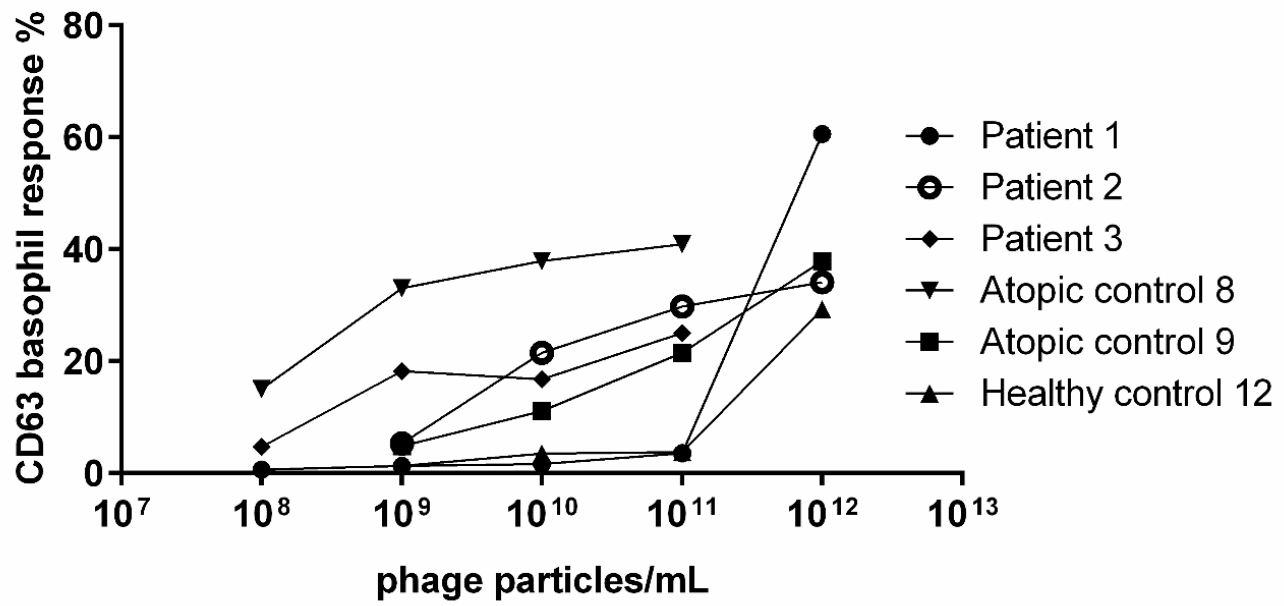
Supplementary Fig. 2. Flow plots of basophil activation tests for patient 1. Stimulation with different concentrations of FMPC S1 and S4.



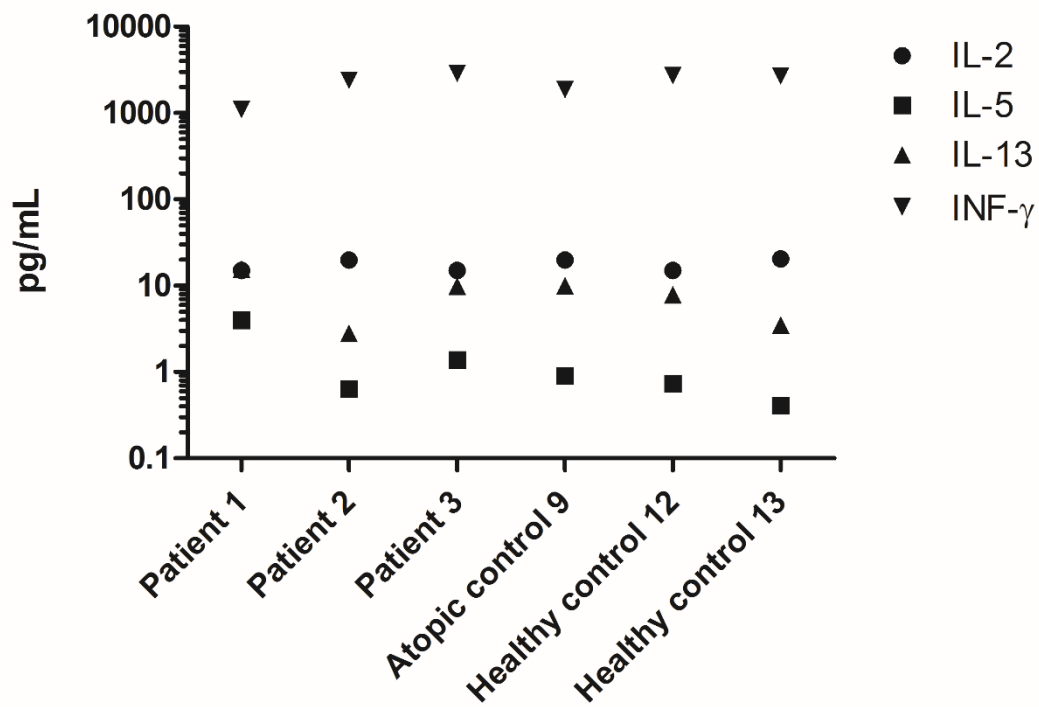
Supplementary Fig. 3. Flow plots of basophil activation tests for patient 1. Stimulation with different concentrations of FMPC S9 and S12.



Supplementary Fig. 4. Flow plots of basophil activation tests for patient 1. Stimulation with different concentrations of FMPC S13 and control phage (wild-type phage).



Supplementary Fig. 5. Basophil activation of six subjects upon stimulation with different concentrations of control phage (wild-type phage).



Supplementary Fig. 6. The cytokine response caused by control phage (wild-type phage).