Allergens displayed on Virus-Like Particles are highly immunogenic but fail to activate human mast cells

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ABSTRACT

Background: The goal of allergen-specific immunotherapy is the induction of protective immune responses in the absence of anaphylactic reactions. We have previously shown that Fel d 1, the major cat allergen, displayed in a repetitive fashion on virus-like particles (VLPs) may fulfill these criteria. Specifically, Fel d 1 on VLPs induced strongly increased protective IgG responses compared to free allergen in mice while anaphylactic reactions were essentially abolished. Here we extend these findings to human mast cells and offer a mechanistic explanation for the reduced anaphylactic activity.

Methods: We differentiated human mast cells in vitro from blood-derived stem cell progenitors and sensitized the cells with a monoclonal Fel d 1-specific IgE. We compared the capability of Fel d 1 to induce mast cell activation in its free form versus displayed on VLPs and we performed allergen binding studies by surface plasmon resonance as well as flow cytometry.

Results: We show that free Fel d 1 induces degranulation of IgE-sensitized mast cells whereas Fel d 1 displayed on VLPs fails to induce mast cell activation. We demonstrate that this inability to activate mast cells is based on a biophysical as well as a biochemical mechanism. Firstly, Fel d 1 on VLPs showed a strongly impaired ability to bind to surface-bound IgE. Secondly, despite residual binding, repetitively displayed allergen on VLPs failed to cause mast cell activation.

Conclusion: These findings indicate that repetitively displaying allergens on VLPs increases their immunogenicity while reducing their potential to cause anaphylactic reactions by essentially eliminating IgE-mediated activation of mast cells.

INTRODUCTION

Allergic reactions are characterized by the induction of IgE responses towards harmless environmental antigens, such as animal dander, pollen, or house dust mite fecal particles (1–3). IgE antibodies are different from other isotypes as they have a very short serum half-life of 5 to 8 hours (4). In contrast, IgE bound to the high-affinity IgE receptor (FcεRI) expressed on mast cells and basophils has a much longer lifespan, as it is protected from degradation (5). Cross-linking of IgE bound to FcεRI by allergens triggers cellular activation causing the release of pre-formed mediators (e.g. histamine), as well as *de novo* synthesis of pro-inflammatory cytokines and chemokines (6,7).

There are a number of different possibilities to treat IgE-mediated allergies. The first choice is the symptom treatment by medications such as antihistamines or corticosteroids. Another approach is the neutralization of IgE by treatment with Omalizumab (8) which has proven effective in severe allergic asthma. Since high doses are required and this treatment is very expensive, more economical approaches for anti-IgE therapies are currently being studied (9).

In contrast to the above-mentioned symptomatic treatments, allergen-specific immunotherapy (SIT) is thought to be disease-modifying. Patients are exposed to multiple doses of the allergen, causing the immune system to tolerate the allergens better (10). There is an ongoing discussion about the most important effector mechanisms causing this state of increased "tolerance" but it is generally accepted that induction of allergen-specific IgG correlates with reduced symptoms and is taken as evidence for successful therapy (11).

We have previously shown that VLPs displaying repetitive arrays of allergens on their surface are potent vaccines to induce high levels of allergen-specific IgG responses and preclinical experiments demonstrated successful immunization against allergy (12). Indeed, a single injection of Fel d 1 displayed on VLPs derived from the bacteriophage Qβ (Qβ-Fel d 1) was able to alleviate symptoms of allergy in mice. Importantly, the high immunogenicity was

paralleled by a strongly reduced ability to activate murine mast cells *in vivo* and *in vitro*. In the present study, we demonstrate that human mast cells, the most important cell type causing local allergic symptoms such as rhinitis or itching of the eye, also show massively reduced responsiveness to Fel d 1 on VLPs compared to free allergen. Surface Plasmon Resonance (SPR) studies and cell binding assays show that the physical and chemical properties of allergens on VLPs are such that interactions with antibodies displayed on a planar surface or displayed by mast cells are reduced. Together, these findings extend previous findings in mice to human mast cells and offer a mechanistic explanation for the strongly reduced potential of allergens displayed on VLPs to cause mast cell activation.

METHODS

Generation of recombinant Fel d 1 and Qβ-Fel d 1

The following nomenclature is used throughout the manuscript: Recombinant monomeric Fel d 1 is the fusion of chain 1 and 2 and corresponds to the natural heterodimer Fel d 1; recombinant dimeric Fel d 1 consisting of 2 recombinant monomeric Fel d 1 molecules linked by a disulfide bridge corresponds to the natural tetramer Fel d 1. Monomeric Fel d 1 was only used to produce the Qβ-Fel d 1 construct. All experiments were performed with recombinant dimeric Fel d 1.

To produce recombinant Fel d 1, a complementary DNA encoding a covalent dimer of chain 1 and chain 2 of Fel d 1 spaced by a 15aa-linker (GGGGS)x3 was generated and fused to a His-tag containing GGCG at the C-terminus. The molecule was expressed in bacteria and purified by a Ni2+ affinity column. In order to separate monomer and dimers from multimers, a size-exclusion chromatography using Superdex 75 pg column (GE Healthcare, Chicago, IL, USA) equilibrated in PBS was performed. VLP derived from the bacteriophage Qβ were expressed in *E. coli* strain JM109 harboring the expression plasmid pQ10 and purified as previously described (13). For coupling, Qβ-VLPs were first reacted at room temperature for 1h with a 5-

fold molar excess of the heterobifunctional chemical cross-linker SMPH. Non-reacting cross-linker was removed by dialysis against 150 mM PBS, pH 7.4. SMPH-derivatized Qβ reacted with 0.2-fold molar excess of monomeric Fel d 1 for 4 h at room temperature while shaking. After covalent coupling, non-coupled Fel d 1 was removed by Amicon Ultra centrifugal filter with a cut-off of 100 kDa.

Immunogenicity in mice

BALB/c mice (Envigo, Huntingdon, UK) were purchased at the age of 6 weeks. The mice were kept at the DKF animal facility, Murtenstrasse 31, Bern. All animals were used for experimentation according to protocols approved by the Swiss Federal Veterinary Office. To test the immunogenicity of the vaccine, 6-week-old naive BALB/c mice were immunized s.c. with either 10 µg Fel d 1 or 10 µg Fel d 1 coupled to Qβ-VLP. For determination of Fel d 1-specific IgG, 96-well Nunc Maxisorp^{TM} ELISA plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with 5 µg Fel d 1 in carbonate buffer at 4°C overnight. After blocking with PBS/0.15% Casein solution for 2 hours, plates were washed five times with PBS/0.05% Tween. Serial dilutions of sera were added to the plates and incubated for 2 hours at room temperature. Plates were then washed five times with PBS/0.05% Tween (PBST). Thereafter, HRP0-labeled goat anti-mouse IgG (The Jackson Laboratory, Bar Harbor, ME, USA) antibodies were incubated at room temperature for 2 hours. ELISAs were developed with TMB (3,3',5,5'-tetramethylbenzidine) and H_2O_2 and stopped with 1 mol/L sulfuric acid. Optical densities were measured at 450 nm. Half-maximal antibody titers are defined as the reciprocal of the dilution leading to half of the OD measured at saturation.

Antibodies

The monoclonal Fel d 1-specific IgE and IgG antibodies used in these experiments have been described previously (14). They have been genetically engineered to be expressed as either

human or mouse antibodies and were produced in CHO cells (Evitria AG, Zürich, Switzerland) and purified by affinity chromatography over a protein L or protein G Sepharose column (GE Healthcare) respectively. The monoclonal Fel d 1-specific IgE F127 was chosen for subsequent activation and binding assays in this study. For activation assays, the mast cells were stained with FITC anti-CD63 (BD Bioscience, NJ, USA) antibodies. Marker expressions were assessed with APC anti-c-kit (Biolegend, San Diego, CA, USA), PE anti-FcεRI (Biolegend) and IgE binding with APC anti-IgE (Miltenyi Biotec, Bergisch Gladbach, Germany). Mast cell binding of Fel d 1 was assessed by staining with the FITC-labelled monoclonal mouse anti-Fel d 1 IgG recognizing the non-overlapping epitope G078 (14).

In vitro generation of human blood-derived mast cells

Buffy coats were purchased from the blood donation center (Swiss Red Cross, Bern, Switzerland). The study was approved by the local ethics committee. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll Paque (GE Healthcare). The general protocol for generating blood-derived human mast cells is published elsewhere (15). Here, mast cell progenitors were isolated using CD133 microbeads (Miltenyi Biotec) and *in vitro* matured over 8 weeks. The cells were cultured in Gibco™ StemPro (Thermo Fisher Scientific) containing supplements, 100ng/ml Gibco™ stem cell factor (Thermo Fisher Scientific) and 40 ng/ml IL-6 (Biolegend). Additionally, the medium contained 10 ng/ml IL-3 (Biolegend) for the first 3 weeks and after 6 weeks, the medium was supplemented with 10 ng/ml IL-4 (Peprotech, Rocky Hill, NJ, USA) and 10% FCS.

Calcium flux

Blood-derived human mast cells were sensitized overnight with 1 µg/ml anti-Fel d 1 F127 IgE and collected the next day. Subsequently, the cells were incubated with the Fluo-4 AM dye (Thermo Scientific) dissolved in Pluronic® F-127 10% solution in water (Thermo Scientific)

and diluted in RPMI-1640 Medium without phenol red (Sigma-Aldrich, St. Louis, Missouri, United States) containing 2.5 mM Probenecid (Sigma-Aldrich) for 30 minutes in a 37°C water bath. The cells were distributed to a Costar Corning 96-well flat bottom black plate (Thermo Scientific) and incubated for 10 minutes at 37°C in the incubator with 5% CO_2. Fel d 1 and Qβ-Fel d 1 were added after 5 minutes of measurement. The measurements were done on the Cytation 5 Imaging Reader (Biotek, Winooski, Vermont, United States) at 37°C for 5 minutes baseline and 15 minutes after allergen challenge at 528 nm wavelength.

Flow cytometry

Imaging flow cytometry was performed using Image Stream^{X}® flow cytometer and the compatible INSPIRE® system software (Merck Millipore, Darmstadt, Germany). Cells were measured at 40x magnification and a flow speed CV below 0.2%. Single cells were gated based on "area" and "aspect ratio" features of the bright-field channel. Focused cells were selected based on the "gradient root-mean-square (RMS)" feature. At least 5'000 single cell events were acquired for every sample and were analyzed using the IDEAS® software (Merck Millipore). Conventional flow cytometry was performed with Guava easyCyte™ (Merck Millipore) or BD FACSCanto™ (BD Biosciences) and analyzed using FLOWJO software (TreeStar Inc.).

Surface plasmon resonance

All surface plasmon resonance experiments were performed with Biacore X100 (GE Healthcare) with HBS-EP+ running buffer (GE Healthcare). The binding of Qβ-Fel d 1 and Fel d 1 to immobilized anti-Fel d 1 antibody was performed using ligand capture method. A CM5 chip was immobilized on both flow cells with 2000 RU Protein A/G and then injected with anti-Fel d 1 IgG F127 for 120 seconds at a constant flow rate of 10μL/minute. The

binding of Qβ-Fel d 1 and Fel d 1 to anti-Fel d 1 IgG F127 was then assessed by injecting Qβ-Fel d 1 (1 nM) and Fel d 1 (60 nM) for 2 minutes on both flow cells at constant buffer flow. An additional buffer control was measured and subtracted from both sample sensorgrams. The ligand capture approach was also used to assess the binding of anti-Fel d 1 IgE to immobilized Qβ-Fel d 1. The CM5 sensor chip was prepared by immobilizing 2000 RU anti-Qβ antibody on both flow cells and then injected with Qβ-Fel d 1 at a concentration of 100 nM on flow cell 2 for 2 minutes at a flow rate of 10µl/minute. The binding to captured Qβ-Fel d 1 was then assessed by injecting anti-Fel d 1 IgE F127 at a concentration of 100 nM for 2 minutes to both flow cells at a flow rate of 10µl/minute.

Statistical Analysis

Statistical tests were performed with GraphPad PRISM 6.0 (GraphPad Software, Inc.). Statistical significance (α=0.05) is displayed as $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****). Groups were analyzed by Two-tailed, student‘s t-test. All data in graphs are displayed as mean ± SEM.

RESULTS

Generation of Fel d 1 displayed on Qβ-VLPs

As previously described, Fel d 1 was expressed recombinantly with a small Cys-containing linker at the C-terminus (12). This free Cys was used to chemically couple the protein to Lys on the surface of the Qβ-VLPs using the cross-linker SMPH. In order to determine the coupling efficiency of Fel d 1 to Qβ, SDS-PAGE bands (Fig 1A) of the coupled products were analyzed by densitometry using Image J (U. S. National Institutes of Health, Bethesda, MD, USA). The density ratio of coupled- and non-coupled Qβ-VLPs revealed that roughly one third of the Qβ subunits were coupled with Fel d 1. As Qβ-VLPs have 180 subunits, this

translates to 60 Fel d 1 molecules per VLP. The *in vivo* immunogenicity of Qβ-Fel d 1 has been shown previously (12). Likewise, the new batch of Qβ-Fel d 1 was tested for the induction of Fel d 1-specific IgG. To this end, BALB/c mice were injected s.c. with either 10 µg Fel d 1, or 10 µg Fel d 1 coupled to Qβ. Fel d 1-specific serum IgG was then measured after 7 and 14 days post-injection. As seen in Fig 1B, Fel d 1-specific IgG titers were only induced by Fel d 1 coupled to Qβ whereas no titer was detected upon free Fel d 1 injection. We have therefore generated an immunogenic vaccine able to induce allergen-specific IgG.

Characterisation of blood-derived mast cells

In order to study the effects of the vaccine on human mast cells, we chose to differentiate human mast cells from CD133+ stem cells isolated from PBMC as previously described (16). The cells were differentiated over 8 weeks in presence of stem cell factor (8 weeks), IL-6 (8weeks), IL-3 (first 3 weeks) and IL-4 (last 2 weeks). After maturation, they visually resembled mast cells when analyzed by microscopy (Fig 2A). The cells were further tested for c-kit and FcεRI expression by flow cytometry; 80-90% of the cells were c-kit positive (Fig2B) and ~90% were positive for FcεRI (Fig2C). Previous studies have shown that these cells exhibit other mast cell characteristics including CD203c expression, presence of tryptase and histamine as well as the absence of monocyte markers CD14 and CD23 (17).

For assessing the ability of the *in vitro* generated mast cells to bind IgE, 8-week maturated mast cells were incubated with monoclonal human IgE F127 antibody against Fel d 1 for 2h at 37°C. Analysis by flow cytometry demonstrated efficient binding of IgE to mast cells (Fig 2D) showing that the cells can be sensitized with anti-Fel d 1 IgE. To test whether the IgE-loaded mast cells can be activated by the allergen, we exposed them to titrated doses of Fel d 1. As seen in Figure 2E, Fel d 1 caused strong activation of mast cells loaded with IgE as assessed by upregulation of degranulation marker CD63. To show that the activated cells are positive for c-kit, we stained CD63 and c-kit on unstimulated or Fel d 1-stimulated mast cells

and analyzed them by flow cytometry. As seen in Figure 2F, the cells expressing CD63 were also positive for c-kit expression. In conclusion, we were able to generate functional human mast cells that could be sensitized with Fel d 1-specific IgE and activated with Fel d 1.

Strongly reduced activation of blood-derived mast cells by Fel d 1 displayed on Qβ-VLPs

We next assessed the anaphylactic potential of the Qβ-Fel d 1 vaccine compared to free Fel d 1 in human mast cells. Therefore, the human mast cells were loaded with IgE and stimulated with titrated doses of Fel d 1 or Fel d 1 displayed on Qβ VLPs. We always compared equal concentrations of Fel d 1 added to the cells, i.e. amounts of Qβ-Fel d 1 were normalized to Fel d 1 content.

We first assessed whether Qβ-Fel d 1 induces CD63 associated degranulation. As shown in Figure 3A, Fel d 1 caused dose-dependent activation of IgE-sensitized mast cells while Qβ-Fel d 1 failed to induce degranulation. To assess whether Qβ-Fel d 1 was able to cause early signs of mast cell activation, we tested levels of intracellular Ca^{2+}. Fel d 1 induced a rapid rise of intracellular Ca^{2+} in IgE loaded mast cells while in contrast, Qβ-Fel d 1 also failed to induce early sign of mast cell activation (Fig 3B).

Since FcεRI mediated activation is accompanied by activation of the Akt/PI3K pathway (18), we investigated intracellular levels of phosphorylated Akt by imaging flow cytometry. To this end, IgE-sensitized mast cells were activated for 5, 10, 30 and 60 minutes at 37°C with either Fel d 1 or Qβ-Fel d 1. The cells were intracellularly stained for phosphorylated Akt (phospho-Akt). Figure 3C shows, that analogous to Ca^{2+} flux, Qβ-Fel d 1 also had no effect on Akt phosphorylation. As seen in Figure 3D, representative images of phospho-Akt^{+} cells after 10 minutes of Fel d 1 challenge clearly show positive staining compared to Qβ-Fel d 1 challenge. In summary, these results indicate that FcεRI signaling is absent upon Qβ-Fel d 1 challenge of

sensitized mast cells. Hence, repetitive arrays of Fel d 1 on VLPs fail to induce signs of mast cell activation.

Reduced binding of Fel d 1 displayed on Qβ-VLPs compared to free Fel d 1

In order to test whether the Qβ-Fel d 1 has inhibitory properties, we mixed titrated doses of Qβ-Fel d 1 with constant amounts of free Fel d 1 and investigated degranulation of IgE-sensitized mast cells. As shown in Figure 4A, Qβ-Fel d 1 did not inhibit mast cell degranulation induced by free Fel d 1 as investigated by anti-CD63 staining and flow cytometry analysis.

To show that recognition of Fel d 1 on Qβ-Fel d 1 by the monoclonal anti-Fel d 1 IgE F127 is not impaired by the coupling, we analyzed binding of IgE to immobilized Qβ-Fel d 1 by Surface Plasmon Resonance (SPR). As seen in Figure 4B, anti-Fel d 1 IgE F127 is able to recognize Fel d 1 displayed on Qβ, suggesting that the recognition of the Fel d 1 epitope is not impeded by the coupling process.

In order to potentially stimulate mast cell activation, soluble Qβ-Fel d 1 has to interact with membrane-bound IgE antibodies. To mimic this situation, we immobilized Fel d 1 specific IgE antibodies on an SPR chip and assessed binding of free Fel d 1 versus Fel d 1 displayed on Qβ-VLPs. As expected, free Fel d 1 bound with high affinity to chip-bound IgE whereas the binding of Fel d 1 on Qβ-VLPs was much weaker as reported by the low increase of resonance units (Fig 4C). Nevertheless, once bound, the interaction was essentially irreversible, as shown by the off-rate that was slow to be measured. Hence, particle-bound Fel d 1 has only restricted access to IgE displayed on a planar surface. To investigate whether the SPR studies translate to the situation on a cellular level, we next performed binding assays with human mast cells. To this end, we incubated IgE-sensitized mast cells with titrated doses of free Fel d 1 or Qβ-Fel d 1 for 30 minutes at 4°C. The cells were then stained at 4°C with FITC-labelled anti-Fel d 1 IgG recognizing a different epitope than anti-Fel d 1 IgE F127.

Consistent with SPR experiments, Fel d 1 displayed on Qβ showed reduced binding to IgE-loaded mast cells (Fig4D). Since we only observed Qβ-Fel d 1 binding to the mast cells at a concentration corresponding to1µg/ml Fel d 1, we next tested whether Qβ-Fel d 1 is able to induce mast cell degranulation at this high concentration. Therefore, we compared the anaphylactic potential of 1µg/ml Fel d 1 displayed on Qβ with 10ng/ml free Fel d 1, the concentration at which Fel d 1 binding was almost undetectable with anti-Fel d 1 staining. As seen in Figure 4E, at this concentration free Fel d 1 still induced degranulation of IgE-sensitized mast cells at 37°C whereas a 100 times more Fel d 1 displayed on Qβ had no effect on degranulation. In summary, we describe two mechanisms that prevent Qβ-Fel d 1-mediated degranulation of IgE-sensitized mast cells. On one hand, Qβ-Fel d 1 binding to IgE displayed on a planar surface or by FcεRI on mast cells is very low, most likely due to slow diffusion of the large particle. On the other hand, once binding occurs, Fel d 1 displayed on Qβ fails to induce degranulation in human mast cells as indicated by the absence of cell activation.

DISCUSSION

We have previously described that recombinant Fel d 1 displayed on a VLP (Qβ-Fel d 1) induces allergen-specific IgG responses capable of preventing Fel d 1-mediated anaphylaxis in a mouse model. Here, we demonstrate that Qβ-Fel d 1 fails to induce degranulation of IgE-sensitized human blood-derived mast cells *in vitro.* We show that this reduced ability to activate mast cells is 1) due to reduced binding of Qβ-Fel d 1 to surface-bound IgE and 2) that Fel d 1 displayed on Qβ fails to trigger FcεRI-mediated degranulation in mast cells. In view of a therapy for cat allergy, the strong immunogenicity and the lack of anaphylactic properties make a strong case for Qβ-Fel d 1 as a potential vaccine.

Cat allergy is one of the most frequent allergic disorders and incidence is still increasing, especially in industrialized countries (19). Most current treatment options against allergies in

general only target symptoms without improving the state of immune dysregulation in allergic patients. The only disease-modifying therapeutic treatment available is allergen-specific immunotherapy (SIT). However, this therapy requires multiple injections of an allergen preparation at modest success rates and is only beneficial for some allergens. Furthermore, no SIT is available for cat allergy. Hence the development of novel, more efficient and more effective therapeutic approaches are required.

In successful SIT, induction of allergen-specific IgG correlates with protection from IgE-mediated hypersensitivity. Specific IgG may alleviate allergic symptoms by 2 proposed mechanisms, mainly by neutralizing the allergen, preventing binding of allergen to cell IgE (competition) and by engaging the inhibitory FcγRIIb receptor (inhibition). VLPs are potent inducers of IgG responses and allergens displayed on VLPs are promising candidates for active desensitization to an allergen. VLPs activate a number of innate humoral defense mechanisms such as natural IgM and complement, which enhance B cell responses elicited by them (20). Coupling allergens to the VLP surface renders allergens equally immunogenic as the underlying VLP and the dominant isotype induced is IgG while essentially no IgE is generated (12).

We have previously established this concept *in vivo* where we demonstrated that a single injection of Fel d 1 coupled to Qβ protected sensitized mice from anaphylactic shock upon Fel d 1 challenge (12). In addition, we could show that mouse effector cells sensitized with anti-Fel d 1 IgE do not degranulate when directly stimulated with the Qβ-Fel d 1 vaccine *in vitro* and *in vivo*. In the current manuscript, we demonstrate that the lack of reactivity to Qβ-Fel d 1 also holds true for human mast cells and delineate mechanisms for the lack of allergic activity.

Fel d 1 displayed on VLPs showed a strongly decreased ability to bind to surface-bound IgE, which offers a partial explanation for the reduced cellular activation. Reduced binding was

not caused by masking of relevant epitopes by chemical coupling of Fel d 1 to Qβ, as free antibodies recognized the allergen well also if Fel d 1 was bound to the VLPs. Furthermore, Qβ-Fel d 1 induced strong IgG responses against the allergen, demonstrating that Fel d 1 epitopes were accessible on the VLP-bound allergen. Taking the dimensions and diffusion of the free Fel d 1 versus Fel d 1 on VLPs into account may be more informative. Assuming that the average mast cell has a diameter of about 10 µm, its surface will amount to around 300 μm^2 or $3x10^8$ nm^2. As shown in supplementary Figure 1, a mast cell has bound approximately 5000 IgE molecules. Therefore, the average area occupied by an IgE molecule is roughly $0.6x10^5$ nm^2. This amounts to an average distance of $(0.6x10^5\ nm^2)^{1/2}$ = 250 nm. The diameter of the VLP plus allergen is about 40 nm, hence more than 6-fold smaller than the average distance between IgE molecules. *In vivo*, this distance is likely even larger, as not all IgE molecules will have the same specificity.

If a VLP-Fel d 1 particle hits a mast cell, the chances are therefore high that it will not readily interact with an IgE molecule. In addition, Fel d 1 on VLPs is not evenly distributed in the fluid but has multiple spots of high concentrations (i.e. the Fel d 1 bound to VLPs) while space in between is empty. As there are roughly 60 Fel d 1 molecules per VLP, the actual concentration of free Fel d 1 is essentially 60 times lower if displayed on VLPs compared to free Fel d 1. Furthermore, according to the Stokes-Einstein equation, the diffusion coefficient D is inversely proportional to the size of a sphere. Assuming a diameter of 4 nm for Fel d 1 and a diameter of 40 nm for Qβ-Fel d 1, D is roughly 10-fold lower for the VLP-Fel d 1 construct. Combined with the 60-fold lower effective free concentration of Fel d 1 on VLPs, a process that is driven by diffusion is about 600-fold lower for VLP-Fel d 1 than for free Fel d 1. Hence, the interaction of Fel d 1 on VLPs is greatly reduced compared to free Fel d 1 for simple physical reasons.

Fel d 1 on VLPs not only interacts less efficiently with IgE bound to mast cells but also fails to activate the FcεRI -mediated signaling cascade. Indeed, under conditions where similar or

higher amounts of Fel d 1 were bound to IgE on mast cells, free Fel d 1 induced strong cellular activation while Fel d 1 on VLPs failed to do so. The block of activation was assessed for 2 independent signaling pathways namely the PI3K/Akt as well as the PLCγ/$Ca2^{+}$ pathway. The inability to trigger these two pathways resulted in a complete failure to stimulate degranulation of mast cells. Why repetitively displayed Fel d 1 fails to trigger FcεRI signaling remains to be further investigated. An attractive hypothesis is that Fel d 1 on VLPs induces large clusters of FcεRI which may not favor initiation of signal transduction. Indeed, it is known that too high concentrations of allergen are inefficient at activating mast cells, a finding that may be based on the same mechanism (21). From a physiological point of view, this may make sense as the IgE-mediated, type II immunity is directed against relatively large parasites, such as nematodes, which have different structural properties than the highly repetitive VLPs.

The reason why Qβ-Fel d 1 is strongly recognized by BCR on B cells but poorly by IgE on mast cells remains to be elucidated. It has been shown that B cell-mediated IgG responses are regulated by epitope density and complement fixation on CD21(22). Therefore, the reason that the VLPs bind to B cells and not to IgE/FcεRI on mast cells might simply be explained by lack of co-receptor expression on mast cells and/or expression of high-density Ig of a single specificity on B cells.

Taken together, we demonstrate here that Fel d 1 displayed on VLPs fails to activate human mast cells and delineate the mechanisms of this failure. These results indicate that allergens displayed on VLPs may have the potential to be safe and effective therapies against allergy.

FIGURE LEGENDS

Fig1: Generation of Qβ-Fel d 1. A) Fel d 1 was coupled to Qβ with 5x excess SMPH cross-linker and various ratios of Fel d 1. Coomassie-stained gel shows Qβ (lane 1), Qβ and cross-

linker SMPH (2), Fel d 1(3) and Fel d 1 coupled to Qβ at a ratio of 0.2x Fel d 1(4). B) BALB/c mice were injected s.c. with a total Fel d 1 amount of 10 µg in free form or coupled to Qβ. Serum anti-Fel d 1 IgG was measured 7 days and 14 days postinjection. Shown are mean ± SEM IgG titers.

Fig2: Human blood-derived mast cells can be sensitized with IgE and activated with Fel d 1. A) Representative microscope image of a May-Grünwald-Giemsa stained mast cell after eight weeks of culture. B) Representative expression of c-kit was measured by flow cytometry displayed as a histogram showing isotype control (gray fill) and anti-c-kit (black). C) Representative expression of FcεRI was measured by flow cytometry displayed as histograms showing isotype control (gray fill) and anti-FcεRI (black). D) Mature mast cells were sensitized with anti-Fel d 1 IgE for 1h at 37°C. IgE binding was measured by flow cytometry displayed as histograms showing an anti-IgE staining of un-primed (gray fill) or IgE-sensitized cells (black). E) Mast cells were sensitized with anti-Fel d 1 IgE for 2h at 37°C and activated with titrated doses of Fel d 1 for 30 minutes at 37°C. The cells were thereafter stained with anti-CD63 at for 15 minutes at RT. $CD63^+$ cells were assessed by flow cytometry, shown are mean ± SEM percentages of activated mast cells from 4 individual donors. F) Representative Scatterplots displaying CD63 versus c-kit fluorescence intensity of IgE-sensitized, unstimulated (left panel) or Fel d 1-stimulated mast cells (right panel).

Fig3: Fel d 1 displayed on VLP does not induce blood-derived mast cell degranulation. Always equal concentrations of Fel d 1 were calculated for free Fel d 1 and Fel d 1 on Qβ for comparing cell activation. A) CD63 activation was assessed by flow cytometry upon addition of titrated doses of Fel d 1 and Qβ-Fel d 1 to IgE-sensitized mast cells. Means ± SEM of CD63+ cells from at least 5 donors are displayed. Two-tailed, student‘s t-test was performed for statistical analysis. B) IgE-sensitized mast cells were stimulated with Fel d 1 or Qβ-Fel d

1 and calcium flux was assessed measuring Fluo-4-AM fluorescence over time by microplate detection at 528nm. C) IgE-sensitized mast cells were stimulated with Fel d 1 or Qβ-Fel d 1 for 5, 10, 30 or 60 minutes, prior to fixation and permeabilization. The cells were then stained intracellularly with anti-phospho-Akt and analyzed by imaging flow cytometry. Shown are mean ± SEM percentages of phospho-Akt $^{+}$ cells of 4 individual donors. Two-tailed, student's t-test was performed for statistical analysis. D) Images from mast cells after 10 minutes stimulated with Fel d 1 (upper panel) or QB-Fel d 1 (lower panel) acquired by imaging flow cytometry. Shown are representative cells displaying bright field and anti-phospho-Akt staining fluorescence.

Fig4: Binding properties of Fel d 1 and QB-Fel d 1

A) IgE-sensitized mast cells were stimulated with Fel d 1 in the presence of titrated doses of Qb-Fel d 1, and CD63 upregulation was assessed by flow cytometry. Mean CD63+ cells ± SEM from 4 individual donors are displayed. B) Surface plasmon resonance was used to assess the binding of a monoclonal anti-Fel d 1 IgE antibody to Qb-Fel d 1 that was immobilized onto a CM5 chip through an anti-Qb capture antibody. C) A CM5 chip displaying captured anti-Fel d 1 F127 via immobilization of Protein A/G was injected with Qb-Fel d 1 (1 nmol/L) and Fel d 1 (60 nmol/L) for 2 minutes to assess the binding of Qb-Fel d 1 and Fel d 1 to anti-Fel d 1 F127. D) IgE-sensitized mast cells were incubated with titrated doses of free Fel d 1 or Qb-Fel d 1 for 30 minutes at 4°C. The cells were then stained with anti-Fel d 1 IgG for 30 minutes at 4°C and analyzed by flow cytometry. Means ± SEM from 4 individual donors are displayed. Two-tailed student's t-test was performed for statistical analysis. E) IgE-sensitized mast cells were incubated with 10 ng/mL Fel d 1 in free form or 1000 ng/ml Fel d 1 displayed on Qb and CD63 upregulation was assessed by flow cytometry. Mean CD63+ cells ± SEM from 4 individual donors are displayed.

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Conflict of interest

M.F.B. declares to be involved in a number of companies developing VLP-based vaccines. F.T. is an employee of Hypopet AG. The other authors declare no further conflict of interests.

Author contributionn

P.E. and MF.B. wrote the manuscript. P.E., M.V., M.F.B., and F.C. designed the study. F.C., P.E., F.S., F.T., and M.V. performed experiments and interpreted data.

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