Immunization of cats to induce neutralizing antibodies against Fel d 1, the major feline allergen in human subjects

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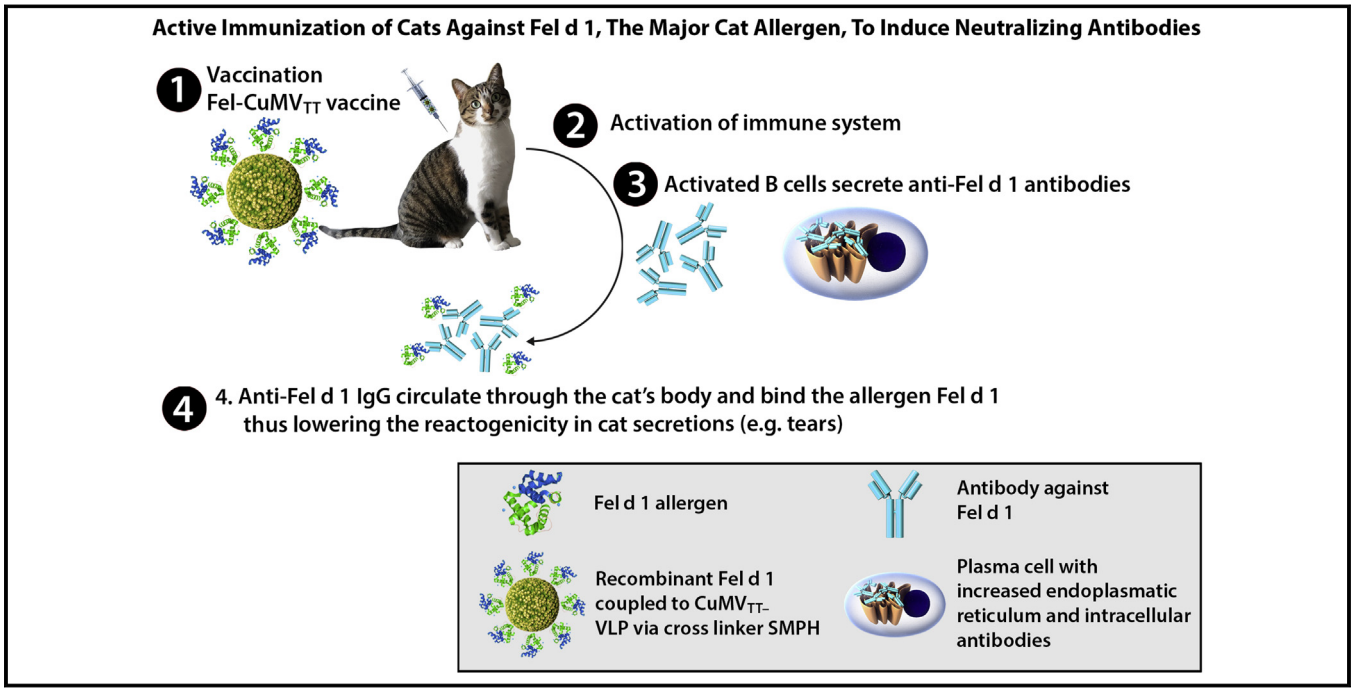
GRAPHICAL ABSTRACT



Background: Cat allergy in human subjects is usually caused by the major cat allergen Fel d 1 and is found in approximately 10% of the Western population. Currently, there is no efficient and safe therapy for cat allergy available. Allergic patients usually try to avoid cats or treat their allergy symptoms.

Objective: We developed a new strategy to treat Fel d 1–induced allergy in human subjects by immunizing cats against their own major allergen, Fel d 1.

Methods: A conjugate vaccine consisting of recombinant Fel d 1 and a virus-like particle derived from the *cucumber mosaic virus* containing the tetanus toxin–derived universal T-cell epitope tt830-843 (CuMV$_{TT}$) was used to immunize cats. A first tolerability and immunogenicity study, including a boost injection, was conducted by using the Fel-CuMV$_{TT}$ vaccine alone or in combination with an adjuvant.

Results: The vaccine was well tolerated and had no overt toxic effect. All cats induced a strong and sustained specific IgG antibody response. The induced anti–Fel d 1 antibodies were of high affinity and exhibited a strong neutralization ability tested both *in vitro* and *in vivo*. A reduction in the endogenous

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allergen level and a reduced allergenicity of tear samples, were observed.
Conclusion: Vaccination of cats with Fel-CuMV$_{TT}$ induces neutralizing antibodies and might result in reduced symptoms of allergic cat owners. Both human subjects and animals could profit from this treatment because allergic cat owners would reduce their risk of developing chronic diseases, such as asthma, and become more tolerant of their cats, which therefore could stay in the households and not need to be relinquished to animal shelters. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

Key words: *Fel d 1, cat allergy, vaccine, antibody, virus-like particle*

Abbreviations used

CP:	Coat protein
CRL:	Charles River Laboratories
CuMV$_{TT}$:	*Cucumber mosaic virus* containing the tetanus toxin–derived universal T-cell epitope tt830-843
HRP:	Horseradish peroxidase
IMAC:	Immobilized metal ion affinity chromatography
LC/ESI/MS/MS:	Liquid chromatography–electrospray ionization–tandem mass spectrometry
OD50:	Reciprocal of the dilution at which half of the OD maximum measured at saturation is reached
SMPH:	Succinimidyl-6-(b-maleimidopropionamido) hexanoate
STT:	Schirmer tear test
VLP:	Virus-like particle

A large number of biological drugs have been developed in the last 30 years for humans; however, they are only now on the horizon of veterinary medicine. Validated targets for humans are now being used to develop mAb therapies for pets to treat cancer and chronic diseases, such as anti-CD52 or anti-CD20 in patients with lymphoma and anti–IL-31 or anti–nerve growth factor in patients with pruritus and chronic pain, respectively. Upon application, mAbs have an immediate effect and mediate symptom relief. However, with respect to their half-life, monthly injections may be necessary to ensure efficacy, with a cost estimation of $5,000 to $50,000 per year in human subjects. In addition, some mAb therapies result in treatment failure by losing their effectiveness because of induction of anti-drug antibodies, and both local and systemic side effects can occur.

An alternative approach is the development of vaccines. Active immunization induces a polyclonal response that increases the possibility of efficacy by recognizing several epitopes and requires 1 to 2 repeated injections per year, with a cost estimate of $200 to $1000. However, the challenge of vaccination is to overcome self-tolerance to induce a strong and sustained antibody response to achieve efficacy in patients with chronic diseases and cancer. Our new conjugate vaccine technology facilitates this by using an immunologically improved plant virus-like particle (VLP) as carrier derived from a modified *cucumber mosaic virus* containing the tetanus toxin–derived universal T-cell epitope tt830-843 (CuMV$_{TT}$).[1]

Compared with viruses, VLPs form icosahedral structures consisting of the viral coat protein (CP) but do not carry genetic information and therefore have no capacity to replicate and only share the viral structural and geometric features. VLPs exhibit a highly repetitive and organized surface, which represents a pathogen-associated structural pattern shared by most viruses but not extracellular host proteins.[2] Furthermore, the CuMV$_{TT}$ VLPs used here carry RNA from the bacterial production strain,[3,4] which is a ligand for Toll-like receptor 7 and 8 and is a pathogen associated-molecular pattern. Thus our VLPs carry both pathogen-associated structural patterns and pathogen-associated molecular patterns, which renders them a suitable carrier platform for antigens by linking them to the surfaces of the VLPs.[5-7] Recently, we have shown that vaccines based on CuMV$_{TT}$ are able to induce antibodies against IL-31 that treat atopic dermatitis in dogs, as well as antibodies against IL-5 that treat insect-bite hypersensitivity in horses.[8,9] Here we extend this platform to immunize cats against their own allergen, Fel d 1.

Cats are found in about 25% of households in Western countries, and allergy to cats is found in approximately 10% of the human population.[10] In addition to Fel d 1–specific IgE antibodies driving type I hypersensitivity, specific T_H cells also contribute to the pathology of the allergic reaction in human subjects, which is known as type IV hypersensitivity, causing recruitment of additional inflammatory cells, such as eosinophils, to mucosal surfaces and other tissues.[11,12] The severity of symptoms ranges from relatively mild rhinitis and conjunctivitis to potentially life-threatening asthmatic exacerbation. Affected patients usually treat their cat allergy symptomatically with antihistamines and corticosteroids, which have limited efficacy, considerable side effects, or both. A second approach is specific immunotherapy involving repeated injections for a minimum of 2 to 3 years with increasing dosages aiming at the induction of IgG antibodies and activation of T_H1 cells, regulatory T cells, or both.[11,13] In recent decades, great efforts have been made to characterize and standardize cat allergens for immunotherapy.[14-16] Moreover, various routes of application, such as the subcutaneous, sublingual, or intralymphatic, were tested for safety and efficacy in mice and human subjects.[14,17,18] Although some very promising results have been observed, none of these therapies have been approved for the market thus far and thus are not available for the treatment of cat allergy in human subjects.

Practical approaches to reduce allergen load in the environment of allergic patients include washing cats, keeping them out of the bedroom, or use of different filter devices, such as high-efficiency particulate air filters or temperature-controlled laminar airflows. However, these efforts are often not implemented long term because there is a lack of commitment and the costs for filters and filtering devices are high and hardly covered by health insurance companies. As a consequence of ineffective therapies, cat owners frequently have to give their cats away to animal shelters,[19] where their fate is often sealed because the shelters often have no choice but to euthanize them.

Within the One Health paradigm, we propose to reduce or prevent cat allergy in human subjects by immunizing cats against their own allergen. Several feline allergens have been identified,[20-23] but the singular importance of Fel d 1 as the major cat allergen has been emphasized in numerous studies showing that approximately 90% of patients with cat allergy have IgE antibodies to this potent allergen.[24] Fel d 1 is constitutively produced by salivary, skin, lacrimal, and perianal glands.[25-29] Male cats exhibit higher levels of the protein and, on castration, reach similar levels compared with female cats.[30] Although some

function in pheromone binding[31] and pelt conditioning has been suggested, the biological function of Fel d 1 remains uncertain. A vaccine targeting Fel d 1 in cats will affect the allergic response at the first possible point of intervention by inducing Fel d 1–specific antibodies in the animal. These antibodies will bind endogenous Fel d 1 and thus decrease or neutralize its allergenic effect in human subjects.

Here we show that vaccination of cats with Fel d 1 displayed on $CuMV_{TT}$-VLPs results in high-titer and long-lasting antibody responses. Antibodies bound to the solvent-exposed surface of Fel d 1 and were able to neutralize the allergen both *in vitro* and *in-vivo*. Hence allergic cat owners may be treated by means of immunization of their cats against Fel d 1.

METHODS

Ethics statement

The study involving human blood from allergic patients was approved by the KEK (Zurich, Switzerland) ethics committee and was conducted under license number ZU-Basophils-001. The immunization studies in cats were performed at Charles River Laboratories (CRL) in Den Bosch, The Netherlands, or Glenamoy, Ireland, and were either approved by the Dutch Animal Welfare Officer and the Ethical Committee (DEC 14-112) or by the Irish Animal Welfare Office (experimental DOH license no. B100/4500). Study procedures were performed in accordance with guidelines of the European Medicines Agency/Committee for Proprietary Medicinal Products (CPMP/SWP/465/95, June 1998) and in accordance with European Union (Protection of Animals used for Scientific Purposes) Regulation 2012 (Statutory Instrument no. 543 of 2012). All mouse experiments were conducted in accordance with ethical principles and guidelines of the Cantonal Veterinary Office, Zurich, Switzerland.

Mice

BALB/c mice were purchased from Harlan (Horst, The Netherlands) and held under enhanced hygienic conditions (Optimaler Hygienischer Bereich in German). Experiments were conducted at the facility in Schlieren, Switzerland, run by the Laboratory Animal Services Center and were in accordance to guidelines approved by the Cantonal Veterinary Office, Zurich, Switzerland.

Cats

Cat studies conducted at CRL (Den Bosch, The Netherlands) included domestic shorthair cats obtained from Liberty Research (Waverly, NY) and acclimatized for 3 weeks. Intact, nonneutered, multiparous, and naive female cats at the age of 1 to 3 years were enrolled in the study. The cat study conducted at CRL, Glenamoy, Ireland, included cats from the CRL Ireland cat colony. They were intact, nonneutered, multiparous, and naive female cats aged 8 to 55 months that had been acclimatized for 7 days.

Cat immunization and sample collection

Two groups of 3 cats received 100 μg of Fel-$CuMT_{TT}$ formulated in 1 mL of PBS 3 times at 3-week intervals in alternating hind limbs intramuscularly. One group of cats received vaccine formulated with adjuvant (15 μg of saponin per cat per dose).

Meta-analysis of the anti–Fel d 1 IgG response included 54 cats of 4 different cat studies. The studies were conducted to investigate various aspects, such as immunogenicity, tolerability, comparison of vaccine batches, and optimal dose finding. Three male and 51 female cats received 3 injections on days 0, 21, and 42 of different doses (3 μg [n = 5], 10 μg [n = 5], 30 μg [n = 5], 100 μg [n = 29, including male cats], 300 μg [n = 5], and 1000 μg [n = 5]) administered subcutaneously.

Serum samples were collected at study days 0, 21, 42, 58, 71, and 85 from all cats and on days 203, 252, 268, and 282 from cats 2, 3, and 5. Blood samples were drawn from the vena jugularis by using serum vacutainers (Greiner Bio-One GmbH, Frickenhausen, Germany). Serum samples were stored at −20°C until analysis.

Tear samples were collected from cats on study days 0, 42, and 84 by using Schirmer tear test (STT) stripes. The STT stripes were placed in the ventrolateral conjunctival fornix for approximately 1 minute. Cats were conscious and tolerated the procedure well. STT stripes were weighed to determine the collected amount of tear fluid.

Size exclusion of recombinant Fel d 1

Pure Fel d 1 solution after immobilized metal ion affinity chromatography (IMAC) purification was characterized by means of size exclusion with a Superdex 75-pg column (GE Healthcare, Chicago, Ill) and an ÄKTA purifier. The analysis was performed with PBS buffer, the storage buffer of recombinant Fel d 1 solution.

Analysis of Fel d 1 by using capture ELISA

ELISA plates were coated overnight at 4°C with an anti–Fel d 1 mouse IgG_1 (clone 6F9 A4 H1; INDOOR Biotechnologies, Charlottesville, Va) as 1 μg/mL in 0.1 mol/L $NaHCO_3$ pH 9.6 buffer and blocked with SuperBlock (Thermo Fisher Scientific, Waltham, Mass) for 2 hours at room temperature. Solutions of 1000 ng/mL natural or recombinant Fel d 1 were prepared and applied to plates, serial diluted, and incubated for 2 hours at room temperature. Bound Fel d 1 protein reacted with a biotinylated mouse IgG1 anti–Fel d 1 mAb 3E4 C4 C10 (INDOOR Biotechnologies) in a 1:1000 dilution in 2% BSA/PBS-Tween for 1 hour at room temperature and further incubated with streptavidin–horseradish peroxidase (HRP) conjugate for 30 minutes at room temperature for detection. Bound enzyme was detected by means of reaction with o-phenylenediamine (OPD; Honeywell, Seelze, Germany) for 7 minutes and stopped by addition of sulfuric acid. PBS-Tween was used as washing buffer. ODs were read with a Tecan Spark 10M ELISA reader (Tecan, Zurich, Switzerland) at 450 nm.

Basophil activation test with blood from patients with cat allergy

The Flow CAST Basophil Activation Test (BÜHLMANN Laboratories, Schöenbuch, Switzerland) was used to determine degranulation of blood-derived basophils by following the producer's instructions. Briefly, stimulation buffer was mixed with EDTA-treated whole blood from a patient with Fel d 1 allergy. An unstimulated blood sample or incubation with a positive (antibody against FcεRI) or unspecific (N-formyl-methionyl-leucyl-phenylalanine, peptide) control were included in the experiment. Serial dilutions of natural and recombinant Fel d 1 were tested for their ability to activate blood-derived basophils. Staining dye containing anti-CCR3 mAb labeled with phycoerythrin and anti-CD63 mAb labeled with fluorescein isothiocyanate were added to the blood samples and incubated at 37°C for 25 minutes. Erythrocytes were lysed, and samples were centrifuged and washed. After centrifugation, cell pellets were resuspended in stimulation buffer and acquired by using a flow cytometer (FACS Fortessa; BD, Franklin Lakes, NJ). Samples were analyzed with FACSDiva software. The percentage of CD63 expression on $CCR3^+$ basophils was determined.

Production of Fel-$CuMV_{TT}$ vaccine

The sequence encoding a fusion of chains 1 and 2 of Fel d 1 was cloned into the plasmid pET42. A histidine tag for purification purposes and a small linker sequence, including a cysteine residue for chemical linkage, were added to the C-terminus of chain 2. The plasmid pET42 was transformed into *Escherichia coli* C2566I (New England Biolabs, Ipswich, Mass), and soluble protein expression was performed at 20°C for 20 hours induced by isopropyl β-D-1-thiogalactopyranoside. Cells were sonicated. Supernatants after centrifugation containing Fel d 1 were collected and purified by using IMAC. After elution with an increased imidazole concentration (250 mmol/L), Fel d 1 was dialyzed against PBS overnight at 4°C.

Expression and purification of $CuMV_{TT}$ are described elsewhere.[1] Briefly, the sequence of the monomer CP of $CuMV_{TT}$ was cloned into the pET28a(+) vector and transformed in *E coli* C2566 cells. Recombinant VLP expression was performed for 20 hours induced by isopropyl β-D-1-thiogalactopyranoside. Cell pellets were sonicated, and insoluble proteins and cell debris were cleared by means of centrifugation. $CuMV_{TT}$-CP was purified by using ammonium sulfate precipitation and ultracentrifugation with a sucrose gradient (20% to 60% sucrose).

Lysine residues of the $CuMV_{TT}$ were reacted with a 7.5× molar excess of the crosslinker succinimidyl-6-(b-maleimidopropionamido)hexanoate (SMPH) for 60 minutes at room temperature to produce the Fel-$CuMV_{TT}$ vaccine. Unreacted SMPH was removed using a PD10 column. Before coupling of Fel d 1 protein with the derivatized $CuMV_{TT}$ particles, Fel d 1 was treated with a 10× molar excess of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP*HCl), a mild reducing agent, to provide access to the cysteine. Derivatized $CuMV_{TT}$ reacted with 2× molar excess of Fel d 1 for 3 hours at room temperature. The vaccine was stored at 4°C until used for vaccination.

Detection of specific IgG antibodies in cat serum by means of ELISA (indirect format)

ELISA plates were coated overnight at 4°C with 1 μg/mL natural Fel d 1 (INDOOR Biotechnology) or 1 μg/mL recombinant Fel d 1 (produced as described herein) in PBS and blocked with SuperBlock (Pierce, Thermo Fisher Scientific) for 2 hours at room temperature. $CuMV_{TT}$ (1 μg/mL) was coated in PBS on ELISA plates overnight at 4°C and blocked with SuperBlock (Pierce) for 2 hours at room temperature to detect anti-VLP IgG. Plates were incubated with serial-diluted study sera for 2 hours at room temperature. Bound cat antibodies were detected with the secondary goat anti-cat IgG HRP (Bethyl Laboratories, Montgomery, Tex) for 1 hour at room temperature. Bound enzyme was detected by means of reaction with OPD (Fluka) for 7 minutes and stopped through addition of sulfuric acid (5% H_2SO_4). PBS-Tween was used as a washing buffer. ODs were recorded with a Tecan Spark 10M ELISA reader at 450 nm. ELISA titers are given as the reciprocals of the dilutions needed to achieve 50% of the OD of the maximal signal measured at saturation (OD50). Geometric mean titers were calculated from all cats of each group.

Detection of specific IgG antibodies in cat serum by means of ELISA (capture format)

ELISA plates were coated overnight at 4°C with 1 μg/mL anti–Fel d 1 human IgG_4 G078[32] in PBS and blocked with SuperBlock (Pierce, Thermo Fisher Scientific) for 2 hours at room temperature. Plates were incubated with either natural (INDOOR Biotechnology) or recombinant Fel d 1 (produced as described herein) in PBS at a concentration of 1 μg/mL for 1 hour at room temperature. Serial-diluted serum samples were applied onto plates for 2 hours at room temperature. The secondary goat anti-cat IgG HRP (Bethyl Laboratories) was added for 1 hour at room temperature. Bound enzyme was detected by means of reaction with OPD (Fluka) for 7 minutes and stopped by addition of sulfuric acid. PBS-Tween was used as washing buffer. ODs were analyzed by using a Tecan Spark 10M ELISA reader at 450 nm. ELISA titers are given as the reciprocals of the dilutions needed to achieve OD50. Geometric mean titers of each time point were calculated from all animals against natural Fel d 1 or recombinant Fel d 1.

Linear epitope mapping of Fel d 1–specific antibodies of cat sera

The epitope mapping analysis of cat sera was performed by PEPperPRINT GmbH (Heidelberg, Germany). The sequence of recombinant Fel d 1 protein was elongated by using neutral GSGSGSG linkers at the C- and N-terminus. The elongated antigen sequence was translated into 15-amino-acid peptides with a peptide-peptide overlap of 14 amino acids. The resulting peptide microarrays contained 187 different peptides printed in duplicate (374 peptide spots) and were framed by additional HA (YPYDVPDYAG) and Flag (DYKDDDDKAS) control peptides. The microarray was blocked with Rockland blocking buffer MB-070 in PBS-Tween. Dilutions of cat sera (1:1000) obtained from the boosting study from days 252 and 312 were applied to the array for 16 hours at 4°C shaking at 140 rpm. Bound antibodies were detected by using goat anti-cat IgG (Fc) DyLight680 (dilution 1:5000) for 45 minutes at room temperature. Arrays were scanned with the LI-COR Odyssey Imaging System (LI-COR, Lincoln, Neb). The structure was created by using PyMol with the Protein Data Bank identifier 2EJN.[31]

Surface plasmon resonance

Surface plasmon resonance measurements were performed with the BIAcore X100 device (GE Healthcare). HBS-EP+ (10 mmol/L HEPES [pH 7.4], 150 mmol/L NaCl, 3 mmol/L EDTA, and 0.005% surfactant P20) was used as the running buffer (flow rate, 10 μL/min). By using an amine coupling kit, monomeric recombinant Fel d 1 produced in our laboratory was coupled to flow cell 2 of a CM5 sensor chip to an immobilization level of 1300 RU (GE Healthcare). Flow cell 1 remained uncoated and served as a control for unspecific binding to the sensor chip surface. Diluted sera from cats 2, 3, and 5 of the immunogenicity study after 2 immunizations obtained on day 43 were then injected for 2 minutes on both flow cells at constant buffer flow. The dissociation rate was measured for another 2 minutes at constant buffer flow. For control purposes, the monoclonal anti–Fel d 1 IgG_1 G078 at a concentration of 50 nmol/L and diluted naive cat serum were injected into the coated chip.

Generation of mAbs against recombinant Fel d 1

By using a mammalian cell display,[33] isolation and generation of 3 mAbs recognizing the nonoverlapping epitopes A044, F127, and G078 on Fel d 1 were recently described.[32]

Neutralization ELISA

ELISA plates were coated with 1 μg/mL anti–Fel d 1 mouse IgG_1 (clone 6F9 A4 H1; INDOOR Biotechnologies) in 0.1 mol/L $NaHCO_3$ (pH 9.6) buffer overnight at 4°C. Plates were blocked with SuperBlock (Thermo Fisher Scientific) for 2 hours at room temperature. Recombinant Fel d 1 at a concentration of 100 ng/mL was incubated for 30 minutes at room temperature either alone, with diluted cat serum samples (1:2 diluted), or with anti–Fel d 1 human IgG_4 A044 at a concentration of 100 ng/mL; applied to ELISA plates; and serial diluted. Bound Fel d 1 protein reacted with a biotinylated mouse IgG_1 anti–Fel d 1 mAb (3E4 C4 C10; INDOOR Biotechnologies) at a 1:1000 dilution in 2% BSA PBS-Tween for 1 hour at room temperature and was further incubated with HRP-SA for 30 minutes at room temperature for detection. Bound enzyme was detected by means of reaction with OPD (Fluka) for 7 minutes and was stopped by addition of sulfuric acid. PBS-Tween was used as washing buffer. ODs were read with a Tecan Spark 10M ELISA reader at 450 nm.

Ear prick tests in mice

BALB/c mice at 8 weeks of age received 5 μg each of the anti–Fel d 1 mouse IgE clones A044 and F127 intravenously to sensitize them to Fel d 1. A couple of hours later, mice received 150 μL of cat serum obtained from an immunogenicity study. Preimmune (day 0) and postimmune (day 63) cat sera were collected from 2 cats that had received 3 injections of 100 μg of Fel-$CuMV_{TT}$ in a 3-week interval on study days 1, 21, and 42. Before transfer into mice, preimmune and postimmune sera were purified by protein A and G (GE Healthcare). One group of mice did not receive cat serum and served as controls.

After 24 hours of cat sera transfer, mice received 200 μL of Evan's blue dye (0.5% wt/vol in PBS). After an hour, anesthetized mice received 10 μg in a volume of 20 μL of recombinant Fel d 1 placed on one ear and pricked through the ear skin by using a lancet. After 45 minutes, mice were killed, and ears were removed to extract the dye as a degree of extravasation. Ears were mixed with 150 μL of 1 mol/L KOH and incubated at 37°C overnight. Then 150 μL of H_3PO_4 (5% in acetone) was added, and the solution was cleared by means of centrifugation. OD values at 595 nm of supernatants were recorded.

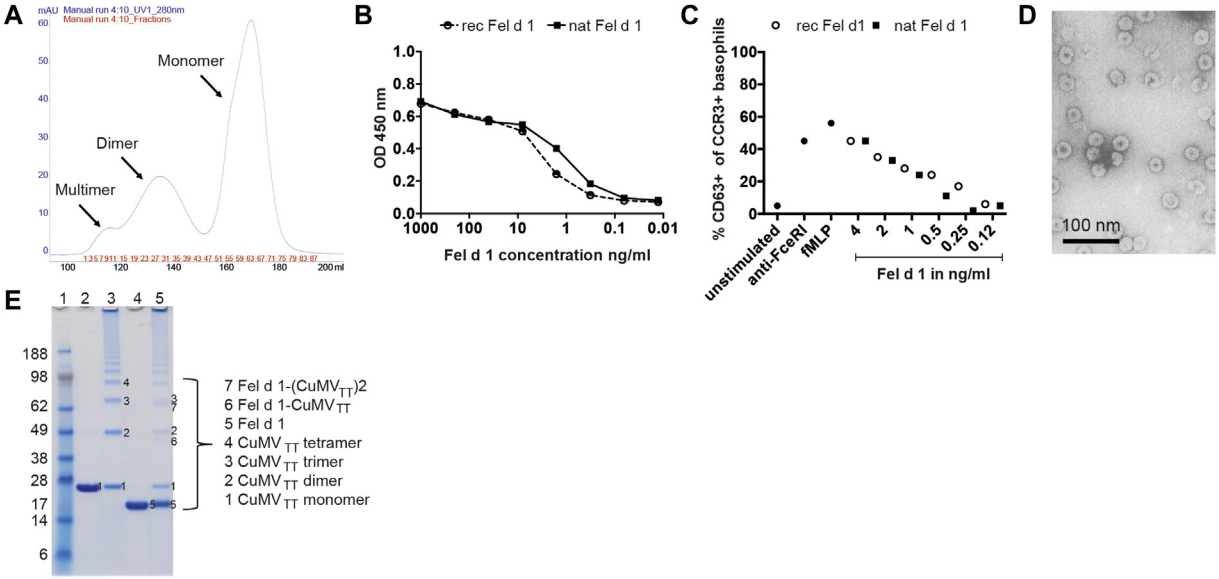

FIG 1. Expression of vaccine intermediates Fel d 1 protein and CuMV$_{TT}$-VLP and Fel-CuMV$_{TT}$ vaccine analysis. **A,** Profile of SEC analysis of recombinantly expressed Fel d 1 after purification with PBS. **B,** Capture ELISA (INDOOR Biotechnology) showing OD values (450 nm) of a series of different concentrations of recombinant *(rec)* and natural *(nat)* Fel d 1. **C,** Basophil Activation Test. Upregulation of the degranulation marker CD63 of blood-derived basophils from patients with cat allergy on incubation with either recombinant or natural Fel d 1 protein was measured. Controls included unstimulated, anti-FcεRI antibody stimulation, and N-formyl-methionyl-leucyl-phenylalanine *(fMLP)*. **D,** Electron microscopic image of purified CuMV$_{TT}$-VLP. **E,** SDS-PAGE analysis (NuPAGE 4-12% Bis-Tris) under denaturing and reducing conditions of the Fel-CuMV$_{TT}$ vaccine. *Lane 1*, Molecular weight marker (Invitrogen SeeBlue Pre-stained); *lane 2*, CuMV$_{TT}$-CP, approximately 24.5-kDa monomer, 5 μg; *lane 3*, CuMV$_{TT}$-CP crosslinked with SMPH, 5 μg; *lane 4*, Fel d 1, 20 kDa, 5 μg; and *lane 5*, Fel-CuMV$_{TT}$ vaccine, 5 μg of VLP or 8.6 μg of total protein.

Quantification of Fel d 1 in tears by using ELISA

ELISA plates were coated overnight at 4°C with an anti–Fel d 1 mouse IgG$_1$ (clone 6F9 A4 H1; INDOOR Biotechnologies) at 1 μg/mL in 0.1 mol/L $NaHCO_3$ (pH 9.6) buffer and blocked with SuperBlock (Thermo) for 2 hours at room temperature. A 1:10 dilution of standard allergen solution was applied to each ELISA plate and 1:2 serial diluted for quantification. Solutions of tear extracts were applied to plates, serial diluted, and incubated for 2 hours at room temperature. Bound Fel d 1 allergen in tear extracts and standard allergen solution reacted with a biotinylated mouse IgG$_1$ anti–Fel d 1 mAb (clone 3E4 C4 C10; INDOOR Biotechnologies) in a 1:1000 dilution in 2% BSA/PBS-Tween for 1 hours at room temperature and was further incubated with HRP-SA for 30 minutes at room temperature for detection. Bound enzyme was detected by means of reaction with OPD (Fluka) for 7 minutes and stopped by addition of sulfuric acid. PBS-Tween was used as washing buffer. The linear range of the standard allergen solution was used for Fel d 1 quantification in cat tear extracts.

Fel d 1 identification and quantification using mass spectrometry

Quantification of Fel d 1 in tear extracts from cats was performed by using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC/ESI/MS/MS). A unique peptide, ALPVVLENAR, of the major cat allergen Fel d 1 located on chain 1 (http://www.uniprot.org/uniprot/P30438) was identified and used for comparison with a synthesized heavy peptide version with a C-terminal (R[13C6; 15N4]). Tear extracts (75 μL) of individual cats were precipitated by mixing with an equal volume of 20% trichloroacetic acid. Pellets were washed twice with cold acetone and dissolved in 10 mmol/L Tris-HCl and 2 mmol/L $CaCl_2$ (pH 8.2) containing 4 ng/μL trypsin (Roche, Mannheim, Germany) and 9.6 fmol/μL of the (R[13C6; 15N4])-labeled Fel d 1 peptide. Microwave (Model Discover; CEM, Matthews, NC)–assisted digestion was performed at 5 W for 30 minutes at 60°C. Samples were dried by means of vacuum centrifugation, dissolved in 0.1% formic acid (Romil, Cambridge, United Kingdom), and analyzed on a nanoAcquity UPLC (Waters, Milford, Mass) connected to a Q Exactive Mass Spectrometer (Thermo Scientific) equipped with a Digital PicoView source (New Objective, Woburn, Mass). Peptides were trapped on a Symmetry C18 trap column (5 μm, 180 μm × 20 mm; Waters) and separated on a BEH300 C18 column (1.7 μm, 75 μm × 150 mm; Waters) at a flow rate of 250 nL/min by using a gradient from 1% solvent B (0.1% formic acid in acetonitrile; Romil)/99% solvent A (0.1% formic acid in water; Romil) to 40% solvent B/60% solvent A within 90 minutes. Mass spectrometer settings were as follows: precursor scan range, 350 to 1500 m/z; resolution, 70,000; maximum injection time, 100 ms; and threshold, 3×10^6. The fragment ion scan range was 200 to 2000 m/z, resolution was 35,000, maximum injection time was 120 ms, and the threshold was 1×10^5.

Raw data files from the mass spectrometer of the Fel d 1 light and heavy peptides were loaded to a software for label-free quantification (Progenesis QI for Proteomics; Waters), and automatic aligning was performed. From each Progenesis peptide ion (default sensitivity in peak picking), a maximum of the top 5 tandem mass spectra were exported by using the charge deconvolution and deisotoping option and a maximum number of 200 peaks per MS/MS. The Mascot generic file (.mgf) was searched with Mascot Server version 2.5.3 (www.matrixscience.com) by using the parameters of 10 ppm for precursor ion mass tolerance and 0.04 Da for fragment ion tolerance. Trypsin was used as the protein-cleaving enzyme, allowing 2 missed cleavages. Carbamidomethylation of cysteine was specified as a fixed modification, and oxidations of methionine and heavy arginine (Arg10) were selected as variable modifications.

A forward and reversed cat database (downloaded on March 18, 2015, from UniProt) concatenated to 260 known mass spectrometric contaminants was searched to evaluate the false discovery rate by using the target-decoy strategy.[34]

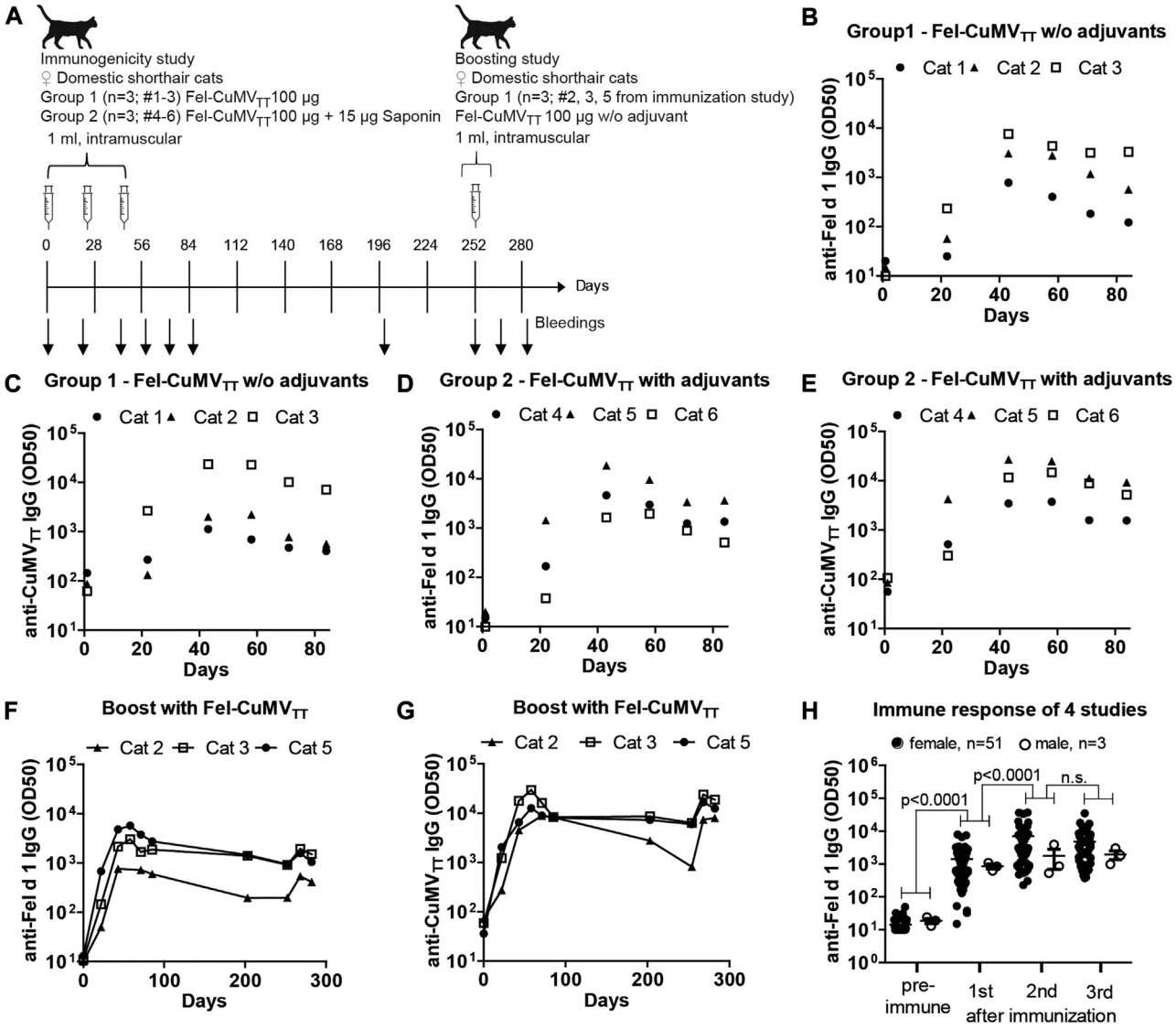

FIG 2. Immunization of cats with Fel-CuMV$_{TT}$ vaccine. **A,** Immunization scheme describing the immunization and subsequent boosting study. **B-H,** Antibody titers determined by using an indirect ELISA are reported as OD50 values. **B,** Serum IgG antibody titers against natural Fel d 1 protein of cats 1 to 3 (group 1). **C,** Serum IgG antibody titers against CuMV$_{TT}$-VLP of cats 1 to 3 (group 1). **D,** Serum IgG antibody titers against natural Fel d 1 protein of cats 4 to 6 (group 2). **E,** Serum IgG antibody titers against CuMV$_{TT}$-VLP for cats 4 to 6 (group 2). **F,** Serum IgG antibody titers against natural Fel d 1 protein of cats 2, 3, and 5. **G,** Serum IgG antibody titers against CuMV$_{TT}$-VLP of cats 2, 3, and 5. **H,** Anti–Fel d 1 IgG in cat sera of 54 cats obtained from 4 different cat studies. Significance was determined by using a 2-tailed paired *t* test comparing all 54 cats before immune serum with first vaccination, first with second vaccination, and second with third vaccination.

Mascot results were exported as XML, and results were imported in Progenesis. Focusing on the abundance of the heavy and light "ALPVVLENAR" peptide (where the concentration of the heavy peptide was *a priori* known) allowed us to estimate the natural (light) concentration of the Fel d 1 peptide for each sample.

Statistical analysis

All data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, Calif), and significance ($P < .05$) was obtained by using paired or unpaired *t* tests.

RESULTS

Generation of a conjugate vaccine targeting Fel d 1

Natural Fel d 1 is a 35- to 39-kDa tetrameric glycoprotein containing 10% to 20% N-linked carbohydrates. It is formed by 2 noncovalently linked heterodimers consisting of 2 chains derived from separate genes. Chain 1 consists of 70 amino acids, and chain 2 consists of 92 amino acids, the latter existing in several truncated forms (78, 85, and 90 amino acids). Three intermolecular disulfide bridges link chain 1 with chain 2, leading to an antiparallel orientation of both chains.[26,35-37] It was previously

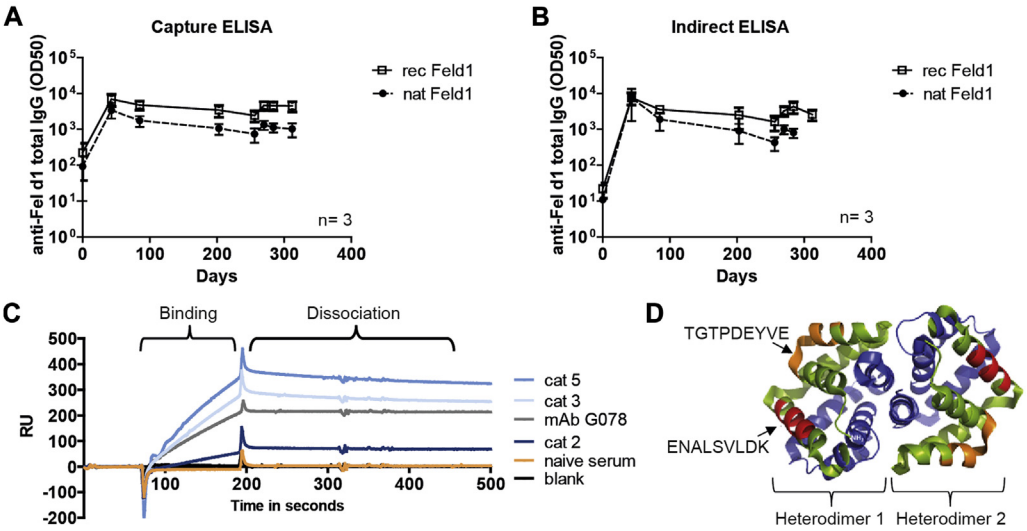

FIG 3. Characterization of antibodies induced by Fel-CuMV$_{TT}$ vaccination in cats. **A** and **B,** Mean OD50 antibody titers measured against natural *(nat)* and recombinant *(rec)* Fel d 1 by using a capture ELISA (Fig 3, *A*) or indirect ELISA (Fig 3, *B*; n = 3). **C,** Interaction of serum antibodies from immunized cats with Fel d 1 antigen measured by using surface plasmon resonance. **D,** Three-dimensional structure of Fel d 1 tetramer (or homodimer) exhibiting the identified epitopes of antibodies found in cat sera upon Fel-CuMV$_{TT}$ vaccination by using linear epitope mapping. Green denotes chain 1 and blue denotes chain 2 of the Fel d 1 heterodimer. The location of the consensus epitope is shown in red, and the minor epitope is shown in orange.

shown that Fel d 1 can be recombinantly expressed as a genetically linked heterodimer with a conserved antigen authenticity[38] that can be conjugated to VLPs.[1,39]

We used the same strategy herein. Fel d 1 protein was recombinantly expressed comprising chain 1 (71 amino acids) fused to the N-terminus of chain 2 (92 amino acids) by a 15-amino-acid linker sequence with modifications at the C–terminus containing a cysteine for coupling to VLPs. Soluble recombinant Fel d 1 protein was expressed in *E coli* and purified by using IMAC. A pure protein was obtained with the majority of monomers, corresponding to the natural dimer caused by the linker and dimers corresponding to natural tetramers (Fig 1, *A*). A commercial capture ELISA using mAbs generated against natural Fel d 1 was performed to test the antigenic authenticity (Fig 1, *B*). We also tested the ability of recombinant Fel d 1 to activate basophils in blood from patients with cat allergy who carry Fel d 1–specific IgE bound to FcεRI by using a basophil activation assay.[40] In comparison with natural Fel d 1 protein, recombinantly expressed Fel d 1 was similarly recognized by mAbs in the capture ELISA (Fig 1, *B*) and could equally stimulate basophils of patients with cat allergy analyzed by upregulation of the exclusive degranulation marker CD63 (Fig 1, *C*).

Generation and characterization of the CuMV$_{TT}$-derived VLPs is described in detail elsewhere.[1] It consists of the CP of the CuMV expressed in *E coli*. The monomers spontaneously assemble into VLPs enclosing RNA from *E coli* during the production process. The icosahedral VLPs have a diameter of approximately 30 nm and consist of 180 copies of CP monomer with a size of 24.5 kDa that leads to a molecular weight of 4430 kDa for 1 VLP (Fig 1, *D* and *E*). The free cysteine in the recombinantly expressed Fel d 1 protein was coupled to lysine residues on the surfaces of VLPs through the crosslinker SMPH to generate a vaccine against Fel d 1. SDS-PAGE analysis of the coupling reaction shows a primary coupling band that corresponds to 1 VLP monomer (24.5 kDa) linked to 1 Fel d 1 protein (20 kDa) with a size of 44.5 kDa (Fig 1, *E*). The coupling leads to a coupling efficiency of 8% that corresponds to 14 molecules of Fel d 1 bound to 1 VLP.

Induction of Fel d 1–specific antibodies upon immunization of cats with Fel-CuMV$_{TT}$

The new vaccine candidate Fel-CuMV$_{TT}$ was tested in naive female domestic shorthair cats in an immunogenicity study (Fig 2, *A*). Before immunization, there were no significant anti–Fel d 1 or anti-CuMV$_{TT}$ IgG levels detectable (Fig 2, *B-E*). Fel d 1–specific IgG was detected on day 22 after a single immunization, and levels increased further after the second immunization on day 22. The third immunization on day 43 maintained the anti–Fel d 1 IgG response at a high level (Fig 2, *B* and *D*).

The anti-CuMV$_{TT}$ IgG response showed similar kinetics as the anti–Fel d 1 response (Fig 2, *C* and *E*). The first immunization significantly increased the anti-CuMV$_{TT}$ IgG response from baseline. Although the second immunization further increased the anti-CuMV$_{TT}$ IgG response, the third vaccination maintained the specific IgG response (until day 252) on a high level (Fig 2, *C* and *E*). Co-administration of a saponin-based adjuvant had little effect on the magnitude and kinetics of the antibody response (Fig 2, *B-E*).

The objective of the booster injection in week 36 was to determine whether late administration of Fel-CuMV$_{TT}$ resulted in maintaining or boosting Fel d 1– and CuMV$_{TT}$-specific antibody levels. Upon booster injection, anti–Fel d 1 IgG and anti-CuMV$_{TT}$ IgG levels in all 3 immunized cats increased to similar levels seen after the first 3 immunizations (Fig 2, *F* and *G*). Of note, the specific antibody titers for Fel d 1 and CuMV$_{TT}$-VLP after the first 3

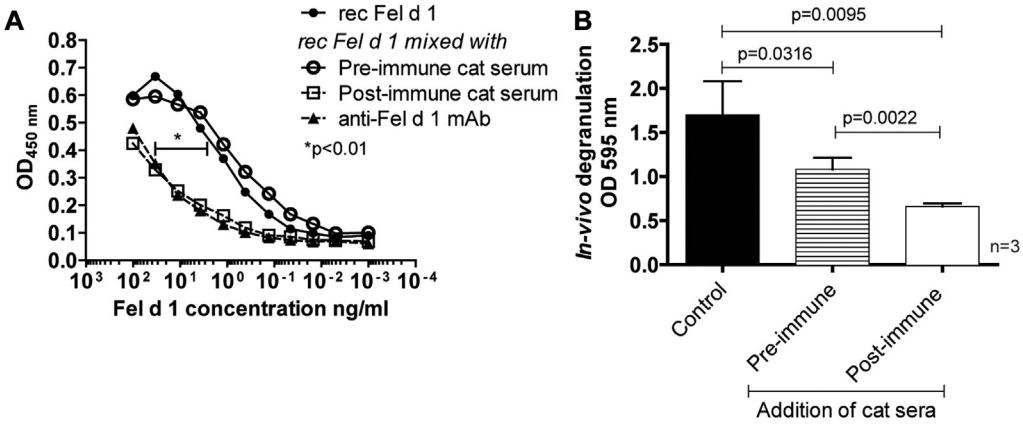

FIG 4. Neutralizing ability of Fel d 1–specific antibodies induced in cats on Fel-CuMV$_{TT}$ vaccination. **A,** Neutralization assay based on a capture ELISA to measure Fel d 1 protein. Recombinant *(rec)* Fel d 1 was assayed either alone or mixed with preimmune (day 0) serum or postimmune (day 42) serum from a cat immunized with 100 μg of Fel-CuMV$_{TT}$ on days 0 and 21, or 100 ng/mL anti–Fel d 1 mAb A044. OD values at 450 nm are shown. *P* values were obtained by using a paired *t* test. **B,** Ear skin prick test. Mice received anti–Fel d 1 IgE clones A044 and F127. Transfer of IgG-purified preimmune or postimmune serum from 2 cats enrolled to a Fel-CuMV$_{TT}$ immunogenicity study were performed. The control group received only the anti–Fel d 1 IgE. Extravasation of Evan's blue dye was quantified by using OD measurements at 595 nm. There were 3 mice in the control group. Mice tested with preimmune sera of 2 cats, n = 6; mice tested with postimmune sera of 2 cats, n = 6. Significance was determined by using a 2-tailed Mann-Whitney test.

immunizations only marginally decreased and stayed at a high level.

A meta-analysis of the anti–Fel d 1 IgG response involving 3 male and 51 female cats from 4 different studies further demonstrates the strong immunogenicity and tolerability of the Fel-CuMV$_{TT}$ vaccine (Fig 2, *H*).

Antibodies induced by Fel-CuMV$_{TT}$ vaccination in cats are highly specific

We characterized the cat anti–Fel d 1 IgG response induced by vaccination by comparing capture (Fig 3, *A*) and indirect (Fig 3, *B*) ELISA formats performed with natural Fel d 1 extracted from fur washes and our recombinantly produced Fel d 1. Antibodies induced in cats after immunization with Fel-CuMV$_{TT}$ recognized coated and captured natural and recombinant versions of Fel d 1 (Fig 3, *A* and *B*). Furthermore, the overall titers and binding kinetics were similar, and the method of antigen display did not influence the binding of cat IgG to antigen, indicating good recognition of natural Fel d 1.

The kinetics of binding and dissociation of serum antibodies with Fel d 1 antigen were analyzed by means of surface plasmon resonance with BIAcore. Preimmune and postimmune (day 43) sera were analyzed for binding to monomeric recombinant Fel d 1, which corresponds to the natural heterodimer Fel d 1 (Fig 3, *C*). Preimmune cat serum did not bind to Fel d 1, whereas serum after 2 injections with Fel-CuMV$_{TT}$ vaccine showed a strong interaction with the antigen. The binding phase of a polyclonal serum in BIAcore experiments reflects the antibody quality and quantity able to bind to immobilized antigen. The BIAcore signal occurred more rapidly for cats 3 and 5 than for cat 2, which is consistent with the anti–Fel d 1 ELISA results in Fig 2, *B* and *C*. In contrast to the binding phase, the dissociation phase reflects the average off-rate of the antibodies and is independent of antibody concentrations. Therefore it is interesting to note that an anti–Fel d 1 mAb (clone G078) with a high affinity and low off-rate[32,41] showed a similar dissociation curve as the cat sera, indicting similar off-rates for the various antibodies.

To further characterize cat antibody binding, we sought to identify their epitope specificity. A linear peptide library encompassing recombinant Fel d 1 was constructed. The library comprised 187 peptides, each 15 amino acids in length, with a 14-amino-acid overlap to the preceding peptide. The peptide microarrays were incubated with immune sera, and peptide binding of cat IgG was identified by means of immunostaining. A strong and clear IgG response to a common epitope was found. This epitope had the consensus motif ENALSVLDK located on chain 1. In addition, a minor epitope with the sequence TGTPDEYVE, also located on chain 1, was identified. Neither epitope was recognized by preimmune sera, but both were recognized by postimmune sera from all cats. The consensus and minor epitopes were mapped onto the 3-dimensional structure of the Fel d 1 tetramer that corresponds to the natural Fel d 1 tetramer (Fig 3, *D*).[1,31] Their positions are consistent with the design of the vaccine and presentation of the Fel d 1 protein on the surface of the VLPs to B cells. Moreover, the epitopes are located on an exposed face of the Fel d 1 tetramer, which is compatible with binding and neutralization of the native allergen Fel d 1.

Neutralization of Fel d 1 by vaccine-induced antibodies

A competition ELISA was performed to assess whether the vaccine-induced antibodies found in cat serum are capable of neutralizing Fel d 1 (Fig 4, *A*). Both sera from immunized cats and a Fel d 1–specific mAb were able to compete to the same extent for binding to soluble Fel d 1 and prevented binding to the antibody coated on the ELISA plate, which resulted in a lower OD value. In contrast, preimmune sera were not able to block binding.

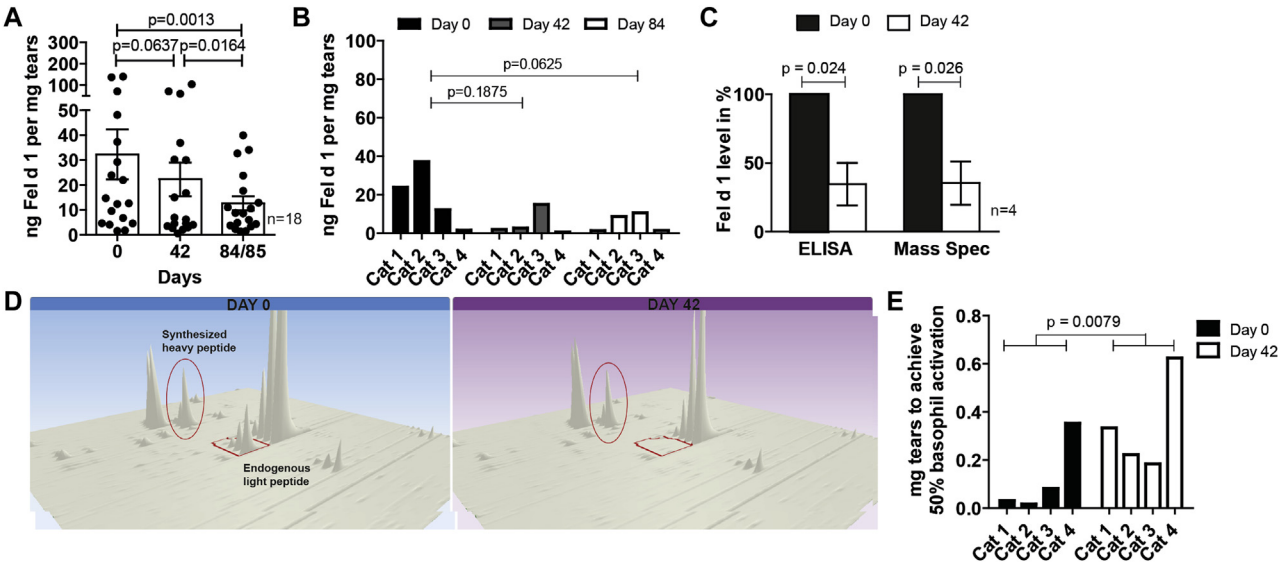

FIG 5. Reduction of Fel d 1 in cat tear extracts. Tears of immunized cats were collected by using STT stripes and analyzed for Fel d 1 levels. **A,** Meta-analysis of Fel d 1 in tear extracts of 18 immunized cats from 3 different studies measured by using a quantitative ELISA (INDOOR Biotechnologies). Fel d 1 levels were normalized to milligrams of tears. Significance was determined by using a 1-way ANOVA, followed by a Wilcoxon matched-pairs signed-rank test. **B,** Measurement of Fel d 1 levels in tear extracts of a group of 4 cats in a previous cat study. Fel d 1 amounts were normalized to milligrams of tears. *P* values were obtained by using a Wilcoxon matched-pairs signed-rank test. **C,** Reduction of Fel d 1 levels in percentages in tear extract solutions before (day 0) and after (day 42) immunization of 4 cats measured by means of ELISA or mass spectrometry. Significance was determined by using a 2-tailed paired *t* test. **D,** Spectrum of the mass spectrometric analysis of light and heavy peptide ALPVVLENAR to quantify Fel d 1 in tear extract solutions collected on day 0 or day 42. **E,** Basophil Activation Test assay was performed with blood from patients with cat allergy mixed with tear extracts from cats from before (day 0) and after (day 42) immunization. Tear extract solutions were diluted to the same Fel d 1 concentrations and tested to determine at which dilution 50% of basophil activation was achieved. The dilution of a certain Fel d 1 concentration that achieved 50% basophil activation was then related to the actual collected amount of the tear sample (milligrams of tears to achieve 50% activation). Significance was obtained by using a paired *t* test.

We further investigated the neutralization capacity of cat Fel d 1–specific antibodies *in vivo* by using an ear prick test in mice pretreated with Evan's blue to visualize the local allergic reaction (Fig 4, *B*).[32] Control mice, which had not received cat serum, had the highest level of local allergic reaction (Fig 4, *B*). Transfer of preimmune cat serum somewhat decreased the reaction; however, transfer of postimmune cat serum clearly reduced the degranulation, indicating neutralization of Fel d 1 *in vivo*.

Taken together, Fel-CuMV$_{TT}$ vaccination induced highly specific Fel d 1 antibodies in cats exhibiting the ability to neutralize Fel d 1.

Reduction of Fel d 1 levels in cat tears

Tears are one of the main sources of Fel d 1 and were therefore analyzed for Fel d 1 levels upon vaccination with Fel-CuMV$_{TT}$. To perform the analysis of tears, we developed a method for collecting and extracting tears. Tears were collected by using STT stripes, extracted in PBS-Tween, and quantified for Fel d 1 levels by means of ELISA in relation to a standard allergen solution. A meta-analysis of 18 cats from 3 independent studies showed a significant mean reduction of Fel d 1 level by a factor of 2.7 (Fig 5, *A*). In addition, one group of 4 cats was randomly selected, and their tears were further analyzed (Fig 5, *B-E*).

The reduction in Fel d 1 levels of tear extracts of those 4 cats measured by means of ELISA (Fig 5, *B*) was confirmed using a second method based on a specially developed mass spectrometric analysis (Fig 5, *C*). For the analysis, a unique peptide, ALPVVLENAR, of the major cat allergen Fel d 1, which was located on chain 1, was identified. This sequence was then used to synthetize a heavy peptide version by modification of the C-terminal arginine (R [13C6; 15N4]). The synthetic heavy peptide of known concentration was spiked into the tear extracts and compared with the light version of the peptide, which was present in tears, as determined by using a combined LC/ESI/MS/MS (Fig 5, *D*).

In addition to quantification of Fel d 1 levels in tears, we also assessed biological activity using a Basophil Activation Test. Based on the quantitative analysis of Fel d 1 levels by means of ELISA, dilutions of tear extracts were prepared containing equivalent amounts of Fel d 1 and incubated with blood from patients with cat allergy. Tear extracts collected after immunization showed reduced reactivity compared with tear extracts collected before immunization (Fig 5, *E*). This demonstrates that vaccination with Fel-CuMV$_{TT}$ induced allergen-specific antibodies in the cat that reduce levels of Fel d 1 in cat secretions (tears) and its ability to elicit allergic reactions.

DISCUSSION

Here we demonstrate successful induction of Fel d 1–specific antibodies in cats with the intent to reduce or prevent allergic

symptoms in human patients with cat allergy. Fel d 1 is a natural and endogenous antigen in cats. Induction of an IgG response against an endogenous protein requires an immunogenic agent that can efficiently overcome B-cell and T_H cell tolerance. Fel-$CuMV_{TT}$ is designed to achieve this by means of repetitive display of the antigen and delivering linked T_H by the foreign $CuMV_{TT}$.

The vaccine was tested in more than 50 cats to date and induced a strong and sustained immune response against Fel d 1 in all animals. No severe clinical signs, skin irritation at the site of injection, or changes in food consumption or behavior were observed. Minor signs, including increased temperature, loss of appetite, or reduced activity, were reversible 24 to 72 hours after immunization. Thus targeting Fel d 1 by means of induction of antibodies through vaccination in cats was judged to be safe and well tolerated. The immune responses against the self-protein Fel d 1 and the foreign $CuMV_{TT}$ were comparable with regard to kinetics and magnitude.

We also tested a saponin-derived adjuvant to potentially increase the specific antibody response. However, the adjuvant only marginally increased antibody levels and also did not affect the kinetics of the response; hence it will not be used in further studies. This is an important point because the use of adjuvants is associated to induce sarcoma in cats and are not normally recommended to be used.[42] The decrease in Fel d 1–specific IgG levels occurred very slowly and titers could be maintained by a booster injection. This indicates a strong B-cell activation with sustained IgG antibody production, even in the absence of an adjuvant.

Binding and neutralization of Fel d 1 by specific antibodies might not be expected to result in toxic effects because they are not likely to negatively affect Fel d 1 production *per se* or result in its complete elimination. This assumption is further supported by the fact that no essential biological role has been found for Fel d 1. Moreover, Fel d 1 levels naturally vary greatly among cats (by more than a factor of 100), and low levels of the molecule, as observed in some neutered cats, are not associated with known diseases.[27,30] Because Fel d 1 is an endogenous protein to which the cat is constantly exposed, it is unlikely to itself have any toxic potential to the cat, particularly when administered in the context of a vaccine.[43]

A potential toxicity resulting from formation of persistent immune complexes comprised of natural Fel d 1 and specific antibodies induced by vaccination and subsequent potential activation of complement and effector cells, which might cause inflammation and tissue damage is low. Fel d 1 is produced by salivary, lacrimal, sebaceous, and perianal glands and is excreted onto the external surfaces of the cat, which are the only known sites of persistence.[44] Fel d 1 has not been found in the circulation, and hence the formation and deposition of immune complexes resulting in activation of complement and effectors cells and subsequent inflammation are not considered likely. The possibility of inducing autoreactive T cells is also considered unlikely because of T-cell tolerance to soluble self-proteins.[45] The T-cell help required for induction of the anti–Fel d 1–induced B-cell response is provided by the $CuMV_{TT}$-VLP. Furthermore, by not including a strong adjuvant in the vaccine formulation, the chances of activating tolerized or anergized potentially autoreactive T cells are minimized. These considerations are supported by the fact that no signs of toxicity have been seen in any of the more than 50 cats immunized thus far. Thus targeting Fel d 1 by induction of antibodies through vaccination in cats can be judged to be safe and well tolerated thus far. Nevertheless, further development of the vaccine Fel-$CuMV_{TT}$ in cats will require a toxicologic assessment by conducting a dedicated animal study.

The goal of vaccinating cats against Fel d 1 is induction of specific antibodies capable of neutralizing the molecule *in situ* (ie, in saliva or tears), rendering cats less allergenic to Fel d 1–sensitive human subjects. The results presented herein provide evidence that high-affinity Fel d 1 antibodies can be induced in cats by means of vaccination and that these antibodies have the ability to neutralize Fel d 1, as demonstrated by *in vitro* and *in vivo* experiments, and also cause a decrease in active Fel d 1 levels in tears, an important source of Fel d 1. Thus vaccination of cats with Fel-$CuMV_{TT}$ can result in reduced symptoms of cat owners, which would not only help the human but also help the cat because of an improved relationship with the owner, which is a new concept within movements like *One Health* (http://www.onehealthinitiative.com).

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Clinical implications: A possible alternative therapy of cat allergy in human subjects is treatment of the cat by vaccination against Fel d 1, the major cat allergen, to reduce its level in cat secretions and its ability to induce an allergic reaction.

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