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ORIGINAL ARTICLE OPEN ACCESS

Evaluation of Inhibitory Activity of Novel Monoclonal Antibodies Against Cat Allergen Fel d 7 and Their Application to Analyse Allergen Extracts

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Received: 24 March 2025 | **Revised:** 27 July 2025 | **Accepted:** 29 August 2025

Funding: This work was supported by Lietuvos Mokslo Taryba (D-20-0929). Research Collaboration Agreement between the University of Salzburg and Amsterdam Medical Center, Austrian Science Funds (FWF) (P34207, I5312).

Keywords: allergen extract | allergy | cat | Fel d 7 | *Felis domesticus* | monoclonal antibody

ABSTRACT

Current characterisation of cat dander/hair extracts used for allergy diagnosis or allergen-specific immunotherapy is mainly standardised towards the major allergen Fel d 1, while other allergens such as the lipocalin Fel d 7 are insufficiently characterised in such allergen products. This study aimed to produce recombinant Fel d 7 (rFel d 7) and murine IgG monoclonal antibodies (mAbs) specific to it for quantification in allergen extracts and assess the potential of the mAbs in inhibiting patients' IgE in functional assays. rFel d 7 was expressed in *E. coli*, purified by Ni-affinity and ion exchange chromatography, and physicochemically characterised by circular dichroism, Fourier-transform infrared spectroscopy, dynamic light scattering and mass spectrometry. Twenty hybridoma cell lines producing Fel d 7-specific mAbs were generated and sandwich ELISA was established for the quantitation of Fel d 7. Six different cat allergen extracts from different manufacturers and prepared from different sources were analysed and the concentration ranged from 0.02 μg/mg to 22.59 μg/mg. A mAb pool recognising non-overlapping epitopes inhibited the binding of human IgE-antigen complex formation (63.7% highest inhibition) and IgE-Fel d 7 cross-linking and consequent degranulation of basophilic cells (57.2% highest inhibition). We demonstrate the vast difference of Fel d 7 content in different cat allergen extracts, highlighting the necessity of improved standardisation of cat allergen extracts. The inhibition results showed that the analysed mAbs effectively inhibit rFel d 7 binding to human IgE, an assay we recommend for assessing the potency of allergen extracts as part of extract standardisation.

Abbreviations: AUC, area-under-the-curve; CD, circular dichroism; DLS, dynamic light scattering; EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; FAB, IgE-facilitated allergen binding; FMO, fluorescence minus one; FTIR, fourier transform infrared; HRP, horseradish peroxidase; huRBL, humanised rat basophil; IPTG, isopropyl β-D-1-thiogalactopyranoside; IUIS, International Union of Immunological Societies; K_d, dissociation constant; LOQ, limit of quantification; mAb, monoclonal antibody; MBP, maltose-binding protein; MS, mass spectrometry; PBS, phosphate buffered saline; PBS-T, PBS with 0.1% Tween-20; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; RBL, rat basophil leukaemia; rFel d 7, recombinant Fel d 7; Rh, hydrodynamic radius; SD, standard deviation; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; WHO, World Health Organization.

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1 | Introduction

Cats are common pets in households and are a prevalent source of inhalant allergens, which can be found even in the public places or in homes without pets [1, 2], making the avoidance of exposure to these allergens almost impossible. Symptoms of sensitisation to cat allergens vary from allergic rhinoconjunctivitis to severe allergic asthma. To cope with the steadily increasing numbers of allergic patients and to provide optimal patient care, including avoidance recommendations or effective immunotherapy [3, 4] a precise allergy diagnosis is necessary. Most commonly, the sensitisation is identified by detecting allergen-specific IgE in a patient's blood serum or performing a skin prick test with allergen extracts. Recent advances in component-resolved diagnostics offer a detailed characterisation of a patient's sensitisation profile, help in distinguishing between cross-reactivity and genuine sensitisation, and provide a high diagnostic accuracy, altogether with the aim of personalised treatment and risk assessment [5]. Despite these advances, allergen extracts are still used for skin prick tests as well as for immunotherapy instead of individual components. Contrary to highly defined allergen components, extracts represent poorly standardised crude materials from the allergen source. For example, allergen extracts show a high variability among manufacturers, have a high batch-to-batch variation, and are derived from different source materials [6–12]. Most of the time, the concentration of individual components in the extracts is either not known or not declared by the manufacturers.

As of 2025, 8 cat allergens have been officially recognised by the World Health Organization (WHO) and International Union of Immunological Societies (IUIS) allergen database (http://www.allergen.org). The first described cat allergen, Fel d 1, was purified and characterised in 1973 [13], and is the most dominant cat allergen with sensitisation higher than 84% among cat allergic patients [14–17]. Low titers of Fel d 1-specific IgE are found in some cat-allergic patients, especially in adults with rhinoconjuctivitis, emphasising the importance of other cat allergens, including Fel d 7, in inducing symptoms [18]. Sensitisation rates to the lipocalin Fel d 7 range between 31%–63% [14–19]. Fel d 7 induces Th2 cytokines upon restimulation of T cells of cat-allergic patients showing the importance of this allergen as Th2 target in cat allergy [20]. Fel d 7 was identified in 2011 and it has a high sequence homology (63%) with the dog allergen Can f 1, and cross-reactivity between these two allergens varies among cat-allergic patients [18, 19]. Lipocalins are found in many animals and they are easily spread into the environment from dander, saliva, sweat and urine [21].

Currently, cat allergen extracts are standardised based on Fel d 1 content, due to its high allergenicity and strong correlation with skin prick tests. Therefore, standardisation of cat allergen extracts is going to continue relying on the content of this allergen, especially after developing novel immunoassays for the measurement of Fel d 1 [22]. Quantification of Fel d 1 was the primary focus in house dust samples [23–26], dog [27] and house dust mite allergen extracts [28] or in in-house cat allergen extracts, alongside the quantification of Fel d 4 [29]. Yet, the analysis of other allergens in cat allergen extracts, such as of the important allergen Fel d 7, remains insufficiently studied. Only a single study attempted the quantification by using a polyclonal antibody in an inhibition ELISA set-up [18]. Therefore, this study aims to fill this gap by providing new insights into its presence in different allergen extracts. A reliable approach to quantify allergen components in the extracts is the use of allergen-specific monoclonal antibodies (mAbs). The aim of this study was to (i) produce recombinant Fel d 7 (rFel d 7) and murine IgG mAbs specific to it, (ii) to perform a detailed physicochemical and immunological characterisation of rFel d 7, (iii) to use the Fel d 7-specific mAbs for quantification of Fel d 7 content in 6 cat extracts from 5 different manufacturers and (iv) to assess the potential of the mAbs in inhibiting patients' IgE in functional assays.

2 | Methods

2.1 | Cell Lines

The mouse myeloma B cell line Sp2/0-Ag14 (ATCC, USA) was used for the generation of hybridomas. Rat basophil leukaemia (RBL) cells humanised by transformation with the human high-affinity IgE receptor FcεRI were used for the mediator release experiments (RBL assay). Epstein–Barr virus (EBV) transformed human B cells that naturally express human FcεRII (CD23) were used to inhibit Fel d 7-IgE complex formation in the IgE-facilitated allergen binding (FAB) assay.

2.2 | Production and Purification of Recombinant Fel d 7

The sequence of cat allergen Fel d 7.0101, termed Fel d 7 in the following, was obtained from the WHO and IUIS allergen database (https://allergen.org/) and UniProt database (https://www.uniprot.org/), accession number E5D2Z5. The synthetic gene encoding Fel d 7 was ordered from Invitrogen (Thermo Fisher Scientific, USA). The DNA fragment encoding the gene (Figure S1) was digested with BamHI and XhoI restriction endonucleases and cloned into digested pET28a(+) vector (Merck Millipore, Germany). The resulting plasmid contained the Fel d 7 sequence, including an N-terminal hexahistidine sequence for purification purposes. The synthesis of rFel d 7 in *E. coli* BL21 (DE3) cells was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the transformed bacteria were cultivated for 2.5 h at 37°C. Cells were pelleted by centrifugation at 1811 × *g* for 15 min at 4°C, washed with rinse buffer (20 mM TRIS, 200 mM NaCl, pH 7.4) and harvested after centrifugation.

E. coli cell pellet was resuspended in Ni-NTA buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8) containing 10 mM imidazole and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were disrupted by ultrasound sonication. The soluble fraction was obtained by centrifugation at 15,000 × *g* for 20 min at 4°C and filtered through a 0.45 μm polyvinylidene difluoride (PVDF) filter. The rFel d 7 was purified by immobilised Ni-affinity chromatography using the ÄKTA start chromatography system (Cytiva, USA) and HisTrap HP 1 mL column (Cytiva, USA). After loading the sample, the column was washed with Ni-NTA buffer with 20 mM imidazole and rFel d 7 was eluted with a 20–500 mM imidazole gradient over

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10 mL. For a second purification step by ion exchange chromatography using the HiTrap Q HP 1 mL column (Cytiva, USA), the buffer of rFel d 7 was exchanged to the ion exchange chromatography buffer (20 mM Tris, pH 7.7) for loading onto the column using the ÄKTA start and the protein was eluted in a 0–1 M NaCl gradient over 10 mL. The concentration of rFel d 7 was determined with NanoDrop 2000c (Thermo Fisher Scientific, USA) spectrophotometer (extinction coefficient 20,065 $M^{-1}\,cm^{-1}$).

2.3 | Physico-Chemical Characterisation of rFel d 7

For the analysis of secondary structural elements of rFel d 7, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy were used. The CD spectrum was recorded by JASCO J-815 spectropolarimeter (Jasco, Japan) at room temperature at a wavelength of 200 to 260 nm. For the measurement, the buffer was exchanged to 10 mM potassium phosphate buffer, pH 7.4 at a final protein concentration of 0.1 mg/mL. For the FTIR measurement, the amide 1 and amide 2 peaks at a wavenumber of 1500 to 1700 cm^{-1} were recorded by the Tensor II FTIR system (Bruker Optics Inc., USA) at 25°C. For sample application, 20 μL of ~2.0 mg/mL of rFel d 7 was loaded using the AquaSpec transmission cell. The data were analysed with the OPUS software 6.0 (Bruker Optics Inc., USA). The second derivative of the amide 1 spectra was calculated using the Savitzky–Golay algorithm (25 smoothing points). For relative quantification of secondary structural elements, the integrated Quant2 method was used and the experimental values for α-helical and β-sheet content were compared to the theoretical values of the PDB (accession number: 8EPV) [30].

The mono-dispersity and aggregation behaviour in solution of rFel d 7 was investigated using the dynamic light scattering (DLS) 802 system (Viscotek Corp., USA). Ten individually recorded measurements were merged and analysed with the OmniSize software (Viscotek Corp.).

The mass spectrometry (MS) analysis was performed with desalted samples on a mass spectrometer (Q-Exactive, Thermo Fisher Scientific, USA) at a flow rate of 1 μL/min. Recorded data were analysed using Protein Deconvolution 2.0 (Thermo Fisher Scientific) and the PEAKS proteomics LC–MS/MS analysis software (Bioinformatics Solutions Inc., Canada).

2.4 | Generation of Monoclonal Antibodies Against rFel d 7

mAbs were generated as previously described [31] by hybridoma technology. Three 6–8-week-old female BALB/c mice were immunised three times every 28 days by subcutaneous injections of 50 μg rFel d 7 using Freund's adjuvant (Thermo Fisher Scientific, USA). Blood samples were collected by tail bleeding before each immunisation and 28 days after the last immunisation. Serum samples were used as a control step to ensure the presence of Fel d 7-specific IgG, determined by indirect enzyme-linked immunosorbent assay (ELISA). The hybridisation was performed as described by Köhler and Milstein [32]. Ten days post-hybridisation, production of Fel d 7-specific IgG by hybridoma cells was screened by indirect ELISA. The selected clones were cultivated, cryopreserved and the culture supernatants containing the mAbs purified by Protein A affinity chromatography using the ÄKTA start system as previously described [33]. The Mouse Immunoglobulin Isotyping ELISA Kit (BD Biosciences, USA) was used to determine the isotypes of the mAbs according to the manufacturers' protocol.

Mice for the immunisation were obtained from Vilnius University, Life Sciences Centre, Institute of Biochemistry, Lithuania (Vet. Approval No LT 59–13-001, LT 60–13-001, LT 61–13-004). Ethical permission to use BALB/c mice in the experiment was obtained from the Lithuanian State Food and Veterinary Agency (Permission No G2-117, issued 11 June 2019). The maintenance of mice and experimental procedures were performed by qualified staff and in accordance with the Directive 2010/63/EU requirements.

2.5 | Conjugation of mAbs to Horseradish Peroxidase

The purified mAbs were conjugated with horseradish peroxidase (HRP) using the periodate method, as previously described [34].

2.6 | Western Blot

Protein samples were first separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride membrane (Carl Roth, Germany) using a semi-dry transfer method. The membrane was blocked overnight with phosphate buffered saline (PBS) containing 2% powdered milk and afterwards incubated with either the mAbs (0.3–10 μg/mL) or sera of patients allergic to cat (1:50 dilution) diluted in PBS with 0.1% Tween-20 (PBS-T) and 2% powdered milk. The detection was performed as previously described [31], using goat anti-mouse IgG (H + L)-HRP (Bio-Rad, USA, catalogue number #1721011, 1:4000 dilution) or mouse anti-human IgE Fc-HRP (SouthernBiotech, USA, catalogue number #9160–05, clone B3102E8, 1:1000 dilution) detection antibodies and 1-Step Tetramethylbenzidine-Blotting Substrate Solution (Thermo Fisher Scientific, USA).

2.7 | ELISA

Indirect, competitive, and sandwich ELISAs were performed as previously described [31] using goat anti-mouse IgG (H + L)-HRP (Bio-Rad, USA, catalogue number #1721011, 1:5000 dilution) or mouse anti-human IgE Fc-HRP (SouthernBiotech, USA, catalogue number #9160–05, clone B3102E8, 1:1000 dilution) detection antibodies.

The indirect ELISA was used for the screening of Fel d 7-specific IgG secreting hybridomas, determination of the apparent dissociation constants (K_d), and cross-reactivity of mAbs to other dog and cat allergens, and for testing the reactivity of rFel d 7 with allergen-specific IgE from sera of patients allergic to cat. Sera of patients with confirmed cat allergy were obtained from

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Plasmalab (USA). For the screening, determination of K_d and cross-reactivity assessment, 96-well plates were coated with 2 μg/mL of rFel d 7 or other cat (MBP-Fel d 1, Fel d 3, MBP-Fel d 4 and Fel d 7) and dog allergens (Can f 2, Can f 5, MBP-Can f 7 and Can f 8) in 50 μL/well; for analysing IgE reactivity, 5 μg/mL were coated. After the blocking step, the plates were incubated with either hybridoma growth medium (50 μL/well), or the purified mAbs (serial dilutions from 0.25–3 μg/mL, 100 μL/well), or serum samples of allergic patients (1:9 dilution, 100 μL/well). The apparent K_d was calculated from a titration curve and defined as a molar concentration of mAb corresponding to the mid-point between the maximum OD450 value and the background. Recombinant cat and dog allergens that were used for the cross-reactivity analysis of mAbs were produced in house (our unpublished data).

The competitive ELISA was performed to identify the overlapping epitopes of the mAbs. The 96-well plates were coated with rFel d 7 (3 μg/mL, 50 μL/well), the wells were blocked and incubated with mAbs (30 μg/mL, 50 μL/well), followed by an incubation with HRP-conjugated mAbs for detection at 1:800–1:5000 dilution.

For the quantification of Fel d 7 in six cat allergen extracts produced by five different manufacturers (MFN#1–5), a sandwich ELISA was established. The 96-well plates were first coated with the capture mAb 10C9 (2 μg/mL, 100 μL/well), and after blocking, incubated with either allergen extract (serial dilutions from 20 to 300 μg/mL, 100 μL/well) or rFel d 7 (serial dilutions from 100 ng/mL, 100 μL/well). For detection, the plates were incubated with the HRP-conjugated mAb 16F1 (1:500 dilution, 100 μL/well).

2.8 | Mediator Release in Humanised Rat Basophil-Based Assay

The mediator release in humanised rat basophil-based (huRBL) assay was performed as previously described [35]. Cells were sensitised with a final dilution of cat-allergic patient serum samples of 1:20 in huRBL cell medium (MEM with Earl's salts, without L-glutamine [Sigma-Aldrich, USA, M8042], 5% FCS, 4 mM L-glutamine and G418 [Sigma-Aldrich, USA, G7513]). Cells were sensitised overnight at 37°C. Serial dilutions (1:10 titration) starting with 1 μg/mL of rFel d 7 were added in a total volume of 100 μL/well to stimulate the sensitised RBL cells. For the inhibition of mediator release in RBL assay, 1 μg/mL of rFel d 7 was preincubated with serial dilutions starting from a concentration of 60 μg/mL of a Fel d 7-specific mAb pool, consisting of equal parts of 10C9, 16F1 and 3D5, or the individual mAbs for 1 h at 37°C to allow for mAb-antigen binding. Following the 1 h incubation, mAb-antigen mixtures were added to previously sensitised huRBL cells (total volume 100 μL/well). Cells were stimulated for 1 h at 37°C, after which the cell supernatant was collected and added to the substrate buffer containing 10 mM 4-MUG, that is, the substrate for the detection of assay read-out, ß-hexosaminidase release, in citrate buffer pH 4.5. After background (untreated cells) subtraction, mediator release was expressed as a percentage of the maximum release control wells (unsensitised and unstimulated cells lysed with 10% Triton X-100 [AppliChem, Germany]).

2.9 | Inhibition of IgE-Facilitated Allergen Binding

FAB assay using EBV-transformed human B cells was performed with 0.1 μg/mL rFel d 7 (5 μL/well) and non-diluted serum samples of patients allergic to cats (20 μL/well) to induce allergen-IgE complex formation, according to the previously published protocol [36]. For the inhibition of complex formation, patients' serum and allergen were simultaneously incubated with either a Fel d 7-specific mAb pool (consisting of equal parts of 10C9, 16F1 and 3D5) or the individual mAbs (used either at a concentration of 8.6 μg/mL or titrated 1:3 from 2.58 to 0 μg/mL, 15 μL/well) for 1 h at 37°C. The mAb concentrations were chosen based on the stoichiometric rFel d 7-to-IgG ratio, with 8.6 μg/mL representing a 10-fold molar excess of the mAbs, 0.86 μg/mL a 1:1 binding stoichiometric ratio, and 2.58 μg/mL and 0.29 μg/mL a 1:3 and 3:1 ratio, respectively, considering that the MW of rFel d 7 is 21.11 kDa and the C [37]. After the inhibition step, EBV cells were added to each well (2×10^7 cells/mL, 5 μL/well) and incubated for 1 h at 4°C. Two wells with EBV cells and serum only were used as a negative control for antigen binding. Cells were washed with PBS, stained with SYTOX Red Dead Cell Stain in APC (Thermo Fisher Scientific, USA, S34859, 1:100 dilution, 100 μL/well), and incubated for 20 min at 4°C. Then, cells were washed with PBS, and Fc blocking reagent TruStain FcX (Biolegend, USA, Cat #101320) was added and incubated for 5 min at 4°C. Lastly, cells were washed with FACS buffer and stained with polyclonal goat anti-human IgE FITC-labelled antibody (KPL/medac GmbH, Germany, Cat #02-10-04, 1:100 dilution) and mouse anti-human CD23 PE-labelled antibody (BD Biosciences, USA, Cat #555711, clone M-L233, 1:40 dilution) for 30 min at 4°C. Fluorescence minus one (FMO) control wells were prepared per stain. Cells were washed and analysed by flow cytometer (CytoFLEX S, Beckman Coulter, USA). FlowJo software (BD Life Sciences, USA) was used for data analysis, and the gates were set according to the FMOs as indicated previously [36]. B cells with IgE-allergen complexes bound to CD23 were gated as CD23+ IgE+ cells of total B cells (Figure S2). Inhibition was expressed as the percentage of decrease in CD23+ IgE+ cells relative to the CD23+ IgE+ cell count of the uninhibited control sample (wells without mAb), representing 0% inhibition.

2.10 | Statistical Analysis

IgE-rFel d 7 binding analysis by ELISA (23 serum samples, three replicate wells) and induction of mediator release analysis by huRBL assay (nine serum samples) results were analysed by GraphPad Prism version 10.12.2 (Dotmatics, USA) and presented as mean ± SD. Area-under-the-curve (AUC) analysis was performed by first transforming the data from the mediator release X = log(X), with the baseline set to the lowest transformed value. The limit of quantification (LOQ) for mediator release was determined by adding the mean baseline values to 10 times the standard deviation (SD) or the

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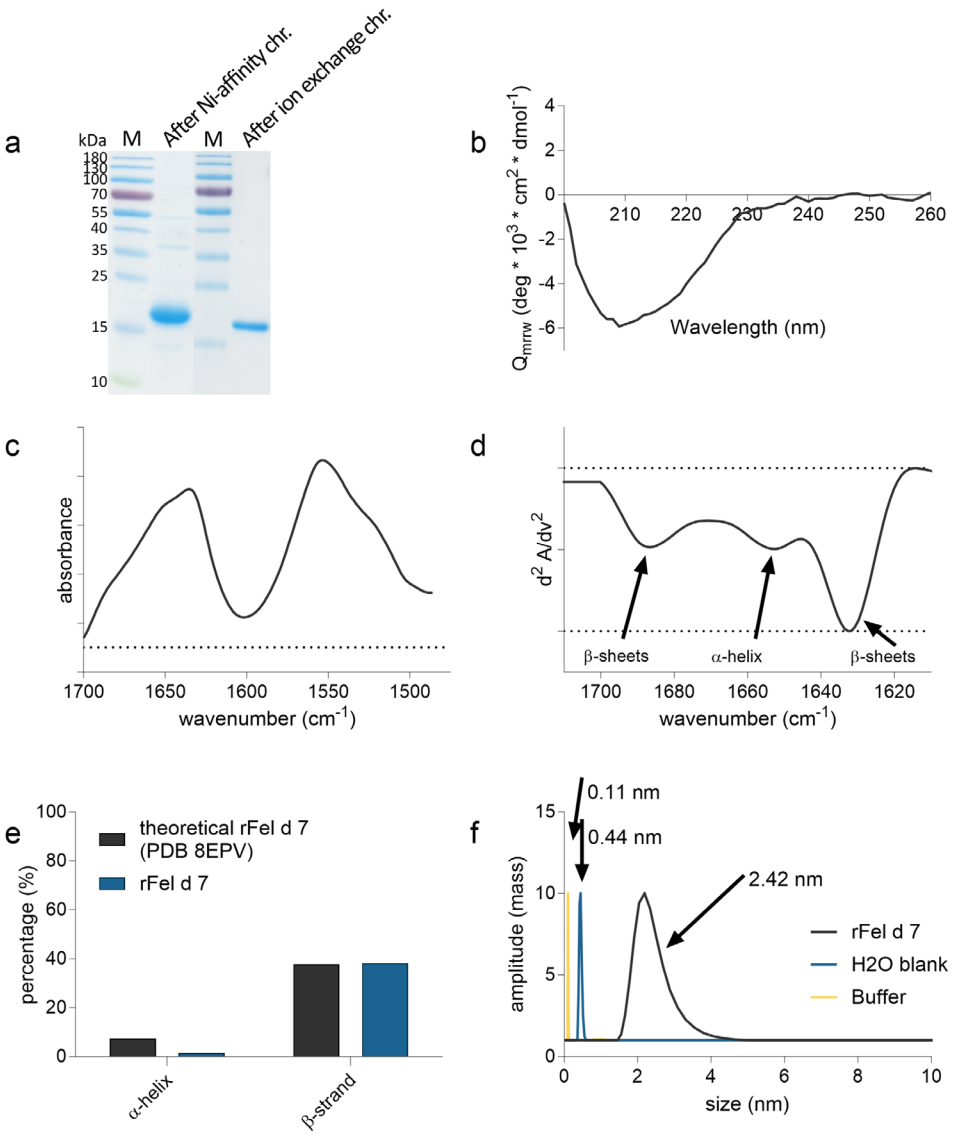

FIGURE 1 | rFel d 7 expression, purification and physicochemical characterisation. SDS-PAGE of rFel d 7 after two-step purification by Ni-affinity chromatography (10 μg) and ion exchange chromatography (2.5 μg), (a). CD spectrum of rFel d 7 recorded at room temperature from 200 to 260 nm (b). Amide I and II (c) and second derivative of amide I (d) IR-spectrum of rFel f 7 measured at 25°C. The percentage of α-helical and β-strand motifs of secondary structural elements obtained experimentally from the IR-spectra was compared to the theoretical values deposited in the PDB, accession number: 8EPV (e). Hydrodynamic radius of rFel d 7 and controls determined by DLS. Data are displayed as mass-weighted distribution of the hydrodynamic radius (f).

baseline. The LOQ was used to define significant mediator release in the huRBL assay. Calculations of the apparent K_d were performed using the OriginPro 8 software (OriginLab, USA) and the results were presented as mean ± SEM (three replicate wells). The standard curve of the optimised sandwich ELISA for quantification of Fel d 7 was analysed by GraphPad Prism and the results were presented as mean ± SD (three replicate wells). Calculation of Fel d 7 concentration in allergen extracts was performed using the SoftMax Pro software (Molecular Devices, USA) and the results were presented as mean ± SD (three replicate wells). Inhibition of IgE-binding to rFel d 7 results by FAB assay (five serum samples) and RBL assay (two serum samples) were analysed by GraphPad Prism and presented as mean ± SD. Repeated measures one-way ANOVA with multiple comparisons was performed and significance was represented with asterisks: $p < 0.05$ (*).

3 | Results

3.1 | Generation and Characterisation of rFel d 7

The coding DNA sequence for the Fel d 7 was inserted into the pET28a(+) vector and the recombinant allergen (188 aa, molecular weight 21.11 kDa) was synthesised in *E. coli*. According to SDS-PAGE analysis, the target protein rFel d 7 was obtained in the soluble fraction of cell lysate. After purification by Ni-affinity chromatography, traces of irrelevant proteins remained. Therefore, to achieve greater purity, rFel d 7 was additionally purified by ion exchange chromatography (Figure 1a).

The MS analysis of the purified rFel d 7 revealed the 100% sequence coverage with the theoretical sequence of Fel d 7 (Figure S3). The CD spectrum revealed a negative peak

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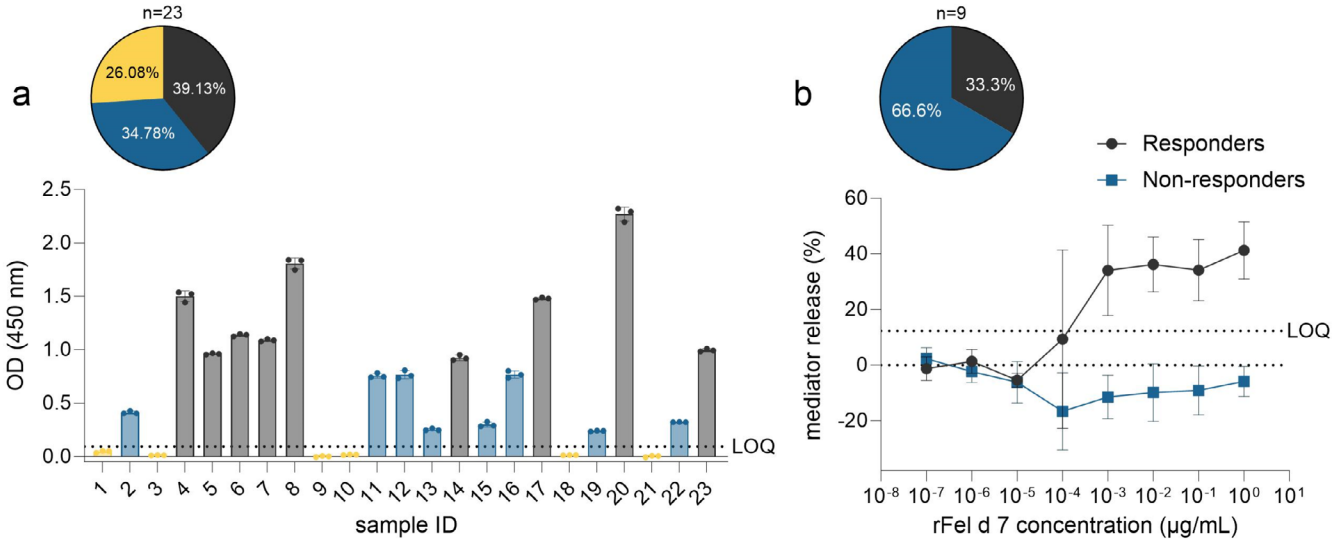

FIGURE 2 | IgE-rFel d 7 binding and induction of mediator release. Reactivity of patients' IgE to rFel d 7 using serums samples from cat-allergic patients was analysed by ELISA (a). Pie chart indicates percentages of non-reactive/below LOQ (yellow), low to mildly reactive (<OD = 0.9, blue) and highly reactive (>OD = 0.9, black) sera. The 9 highly reactive serum samples in the ELISA (black bars) were further screened in the huRBL assay to trigger cell degranulation upon rFel d 7 stimulation (b). The serum samples ($n = 9$) were diluted 1:20 in huRBL cell medium and stimulated the following day with a dilution series of rFel d 7, starting with 1 µg/mL. Pie chart indicates the percentage of responders (black) and non-responders (blue). LOQ is represented with a dashed line and was determined either based on the background values (secondary antibody control) or on untreated cells for ELISA and huRBL assay, respectively. Error bars represent standard deviation.

(minimum) at ~210 nm, indicative of a folded protein with a β-sheet-dominated secondary structure (Figure 1b). The secondary structural elements were further confirmed by the FTIR spectra showing a high percentage of β-sheets but also a few α-helical structures (Figure 1c,d). When comparing the experimental values of the secondary structural elements obtained by FTIR with the theoretical values from PDB (8EPV), a high structural similarity between rFel d 7 and the structure deposited on PDB was revealed with 1.5 vs. 7.4 for α-helix and 38.2 vs. 37.7 for β-sheet content, respectively (Figure 1e). The purified rFel d 7 was 100% monomeric and had a hydrodynamic radius (Rh) of 2.42 nm, which would correspond to a protein with a MW of ~26 kDa and, thus, comparable to the calculated value of 21.11 kDa (Figure 1f).

To assess the allergenicity of rFel d 7 compared to natural Fel d 7, the reactivity of rFel d 7 with IgE of serum specimens obtained from cat-allergic patients was investigated in ELISA and huRBL assay. In total, 23 different serum samples were tested by commercial allergy diagnostic systems, such as Phadia (Thermo Fisher Scientific, USA), to confirm their reactivity with cat allergen extract as reported by the supplier (Table S1). According to the sample description provided by the supplier, the levels of cat extract-specific IgE ranged between 18.54 and 100 kU/L. Of the 23 sera, nine were CAP class 4, nine CAP class 5 and five sera CAP class 6. These samples were analysed by an indirect ELISA for the reactivity with rFel d 7 (Figure 2a). Nine serum samples (39.3%) were highly reactive with rFel d 7 (OD > 0.9) in ELISA and seven of them showed the reactivity with rFel d 7 by WB (Figure S4). Interestingly, nine serum samples that responded well in the ELISA were equally distributed among CAP class 4–6 (cat extract-specific IgE), with two serum samples in CAP class 4, four in CAP class 5 and three in CAP class 6. Eight samples (34.78%) showed low to mild reactivity (OD 0.1–0.9) and six samples (26.08%) were non-reactive (OD < 0.1, below LOQ) with rFel d 7 in ELISA. A comparison of the WB and ELISA reactivity patterns across the serum samples revealed that the results are largely consistent between the two methods; in 13 cases, the level of reactivity observed in WB directly corresponded with the reactivity detected by ELISA (Table S1). However, a few discrepancies were noted where samples exhibited low reactivity in one assay but no reactivity in the other, indicating some variability between the two detection techniques. This variability may reflect the higher sensitivity of ELISA, as seen in cases where low reactivity was detected mostly by ELISA but not by WB.

Selected nine serum samples with the highest reactivity in ELISA were additionally screened in the huRBL assay (Figure 2b, Figure S5) to determine rFel d 7 allergenicity on huRBL cells sensitised with cat-allergic patient serum samples. Three of nine tested serum samples (33.3%, ID 8, 14 and 20) resulted in mediator release (~40% maximum release) upon stimulation with rFel d 7 when used to sensitise the huRBL cells. Those huRBL cells sensitised with responder serum samples were already activated on average by an antigen concentration as low as 1 ng/mL (range: 0.1–10 ng/mL) of rFel d 7. These results indicate that the rFel d 7 antigenic structure conforms to the native allergen enough to be recognised by IgE from cat-allergic patient sera and induce mediator release.

3.2 | Generation and Characterisation of Fel d 7-Specific mAbs

Using the hybridoma technology, Fel d 7-specific murine mAbs were generated. During the immunisation procedure, titers of

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	Clone	K_d (pM)	IgG subtype	WB reactivity
1	13A3	60.3 ± 1.9		++
2	5C3	68.1 ± 1.7		++
3	3D5	86.5 ± 2.2		++
4	14C4	87.2 ± 0.8	IgG1 κ	+
5	2D8	87.5 ± 3.4		+
6	12F10	90.6 ± 4.6		+
7	5A8	108.5 ± 3.2		++
8	10C9	109.8 ± 1.9		++
9	16F1	130.5 ± 3.2	IgG2a κ	++
10	9D9	132.6 ± 1.1		++
11	3A5	135.9 ± 1.7		+
12	1D11	259.7 ± 4.1		++
13	5G5	296.6 ± 9.9		+
14	3B7	365.8 ± 12.1		++
15	6E10	452.1 ± 2.8	IgG1 κ	++
16	4H1	463.1 ± 18.9		+
17	14A7	540.8 ± 5.8		+
18	9A4	1409.9 ± 50.2		+
19	21H3	>2000		+
20	5F2	>2000		+

b

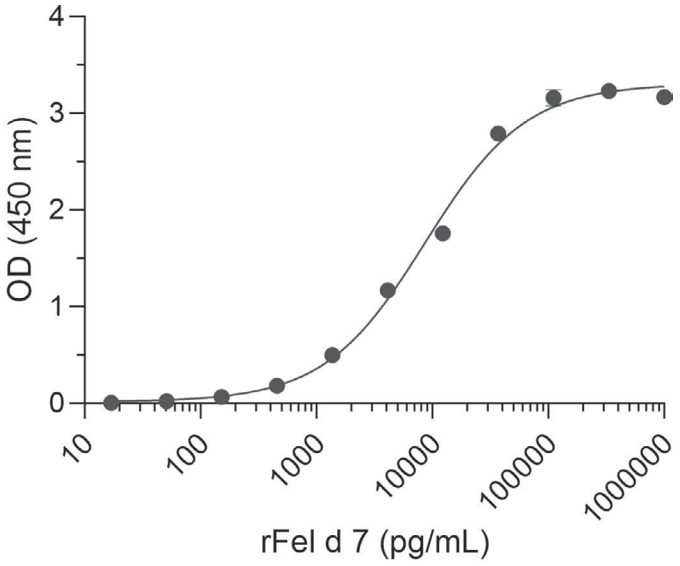

FIGURE 3 | Characterisation of Fel d 7-specific mAbs and development of a sandwich ELISA for the quantification of Fel d 7. Table with characteristics of Fel d 7-specific mAbs, including binding affinity (K_d), IgG heavy and light chain specifications and reactivity in WB (a). Antibody pair used for the sandwich ELISA is highlighted in grey. Standard curve of sandwich ELISA for the quantification of Fel d 7 using the mAbs 10C9 and the HRP-conjugated 16F1 (b).

Fel d 7-specific IgG in blood samples of immunised mice were determined, and the results showed high immunogenicity of the allergen (data not shown). After the hybridisation, hybrid clones were screened by an indirect ELISA for reactivity to rFel d 7 and an irrelevant protein (maltose-binding protein, MBP) as a negative control. Twenty stable hybridoma cell lines producing Fel d 7-specific mAbs of IgG isotype were generated, all with κ light chain. Nineteen (95%) of them were of the IgG1 subtype and one IgG2a (Figure 3a). The reactivity of the mAbs with SDS-denatured rFel d 7 was analysed by WB. All mAbs were reactive with rFel d 7 in WB but with different intensity (summarised in Figure 3a, Figure S6 and data not shown). No reactivity with the negative control (*E. coli* proteins and MBP) was determined.

To exclude that the produced mAbs might interact with other allergens, they were tested in an indirect ELISA using recombinant cat and dog allergens, including MBP-Fel d 1, Fel d 3, MBP-Fel d 4, Fel d 7 and dog allergens (Can f 2, Can f 5, MBP-Can f 7 and Can f 8). Recombinant MBP was used as a negative control. No cross-reactivity of the mAbs with any cat and dog allergens other than rFel d 7 was observed (Figure S7 and data not shown), not even with Fel d 4, also belonging to the protein family of lipocalins. These data indicate that all generated mAbs are highly target-specific. The binding affinity of mAbs to rFel d 7 was analysed by indirect ELISA by determining the apparent K_d. The calculated K_d values of 18 mAbs ranged from 60.3 to 1409.9 pM (Figure 3a) indicating a high binding affinity, while two mAbs (21H3 and 5F2) showed lower binding affinity with K_d values > 2 nM. The results of mAb testing show that the majority of mAbs bind specifically to Fel d 7 with high affinity; therefore, they can be applied for its quantitation and inhibition assays.

3.3 | Application of Fel d 7-Specific mAbs for the Analysis of Cat Allergen Extracts

Since the results of the mAb screening showed that the majority of the antibodies bind with a very high affinity specifically to Fel d 7, a sandwich ELISA was established for the quantitation of Fel d 7 in cat allergen extracts. In this respect, 10 mAbs with the highest K_d (Figure 3a, no. 1–10) were conjugated with HRP using the periodate method, and different pairs of conjugated and non-conjugated mAbs were screened. Non-competing antibody pairs recognising different epitopes were selected by using a competitive ELISA. In total, 41 mAb pairs that recognise non-overlapping epitopes were identified (Figure S8). These pairs were tested in a sandwich ELISA with rFel d 7. The best target recognition/highest detection sensitivity based on the rFel d 7 titration curve was obtained with 10C9 as a capture and 16F1 as a detection (HRP-conjugated) mAb (Figure 3b). Then, the sandwich ELISA was optimised for the highest sensitivity, evaluating different ELISA conditions: plate type, capture mAb 10C9 concentration, detection mAb 16F1 dilution, immobilisation, and blocking solutions. OD values for concentrations at 152.4 pg/mL or higher in the optimised sandwich ELISA for Fel d 7 were above the LOQ (LOQ: OD = 0.0452).

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a

MNF	Source	Fel d 7 (μg/mg)†	WB
#1	Dander	7.89 ± 0.19	+
#2	Epithelium	3.09 ± 0.11	+
#3	Hair	1.19 ± 0.01	–
#4	Epithelium	0.03 ± 0.0003	–
#5	Epithelium	0.02 ± 0.0002	–
#5	Hair	22.59 ± 0.66	+

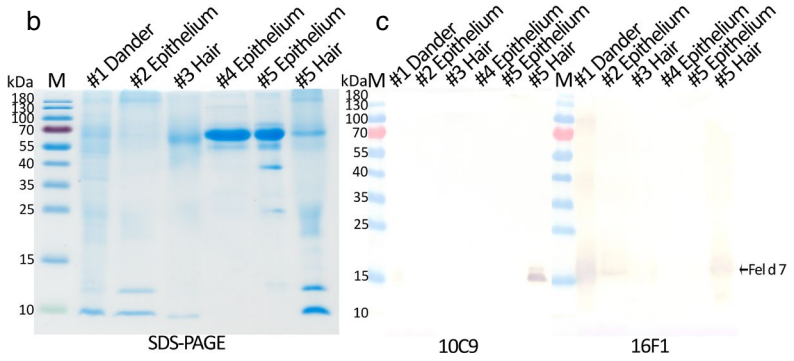

FIGURE 4 | Investigation of cat allergen extracts using Fel d 7-specific mAbs. Quantification of Fel d 7 of cat allergen extracts from different manufacturers (MNF) of sandwich ELISA and WB using Fel d 7-specific mAbs (a). Analysis of cat allergen extracts (5 μg per slot) by SDS-PAGE (b) and WB (c) using the Fel d 7-specific mAbs 10C9 and 16F1. †Allergen mass (μg) per 1 mg of total protein mass of extract.

Six different commercially available cat allergen extracts from five different manufacturers (MNF#1–5) and three different allergen sources (dander, epithelium, hair) were analysed using Fel d 7-specific mAbs (Figure 4a). The precise concentration (mass fraction) of Fel d 7 in the extracts was calculated by sandwich ELISA. Fel d 7 concentration highly varied among different allergen extracts, with the highest being 22.59 ± 0.66 μg/mg in MNF#5 cat hair allergen extract and the lowest 0.03 ± 0.0003 μg/mg and 0.02 ± 0.0002 μg/mg in cat epithelium allergen extracts from MNF#4 and MNF#5 respectively. The variations were observed between both MNF and allergen sources. Even though there was the highest Fel d 7 concentration in hair allergen extract of MNF#5, in hair allergen extract of MNF#3, the concentration of Fel d 7 was significantly lower (1.19 ± 0.01 μg/mg).

Differences in protein content among different allergen extracts were clearly seen when analysed by SDS-PAGE (Figure 4b). A pronounced band was observed between 70 kDa and 100 kDa in MNF#4 and MNF#5 cat epithelium allergen extracts, most likely belonging to the serum albumin Fel d 2 with 69 kDa. Other protein bands were almost invisible in these two samples, especially in the range of 10 and 25 kDa corresponding to Fel d 7. The results obtained by sandwich ELISA were supported by WB using the same mAb clones, 10C9 and 16F1 (Figure 4c). Fel d 7 was detectable in MNF#1 dander, MNF#2 epithelium and MNF#5 hair allergen extracts with clone 16F1, but only in MNF#5 hair allergen extracts by clone 10C9. Based on the sandwich ELISA results, these three allergen extracts contained the highest amount of Fel d 7, highlighting the consistency of ELISA and WB results.

3.4 | Inhibition of Functional IgE-rFel d 7 Binding by Fel d 7-Specific mAbs

Fel d 7-specific mAbs were analysed for their ability to mask human IgE epitopes on rFel d 7 by using the FAB and the huRBL assay. In the FAB assay, the inhibition of rFel d 7 binding to IgE from serum samples of cat-allergic patients was analysed with the mAbs 10C9, 16F1 and 3D5, and a pool thereof. The three antibodies were chosen because 3D5 had a high affinity to rFel d 7, and 10C9 and 16F1 because of their performance in the sandwich ELISA. Further, according to the competitive ELISA, all three mAbs recognised different epitopes (Figure S7). Here, the capacity of the mAbs to competitively inhibit IgE–rFel d 7 complex formation was tested. Nine serum samples that were highly reactive with rFel d 7 in the indirect ELISA were analysed. Five serum samples (ID 4, 5, 6, 8, 17) demonstrated a strong response after screening in the FAB assay (data not shown). On average, the mAb pool competitively inhibited the binding of IgE-antigen complex formation in a concentration-dependent manner (Figure 5a). Mean percentage of inhibition by the mAb pool reached 63.7% at the highest concentration (8.6 μg/mL corresponding to a 10-fold molar excess to rFel d 7). When comparing the inhibition capacity of the mAb pool and individual mAbs to the uninhibited condition (average 27.12% of CD23+ IgE+ EBVs), the pool with 7.6% was the only condition that significantly reduced the binding of IgE-antigen complexed to the CD23 receptor ($p < 0.05$, Figure 5b). After calculating the percentage of inhibition and comparing all the inhibition conditions, the mAb pool (mean 72.0%) showed a statistically higher inhibition capacity than inhibition with the individual mAb 3D5 (mean 9.8%, $p < 0.05$, Figure 5c). The highest percentage of inhibition of the individual mAbs was observed for 16F1 (mean 41.0%), although the difference to 3D5 and 10C9 was not significant.

The potential of the mAbs to inhibit functional IgE-allergen binding was further investigated in the huRBL assay (Figure 5d,e). Here, the inhibition was achieved with the mAb pool (10C9, 16F1 and 3D5), and the serum samples ID8 and ID20 were chosen to sensitise the huRBL cells as these serum samples were found to be responsive to rFel d 7 in the previous huRBL assays (Figure 2b). The results showed that the mAb pool was able to inhibit rFel d 7 binding to IgE and subsequent degranulation of huRBL cells at the two highest concentrations 6 and 60 μg/mL, corresponding to a ~10:1 and ~1:1 mAb:allergen ratio, respectively. The percentage of inhibition was calculated for the two serum samples and a mean percentage of inhibition of 57.2%, 47.5% and 18.0% was achieved at 60, 6 and 0.6 μg/mL concentration of the mAb pool, respectively (Figure 5e). At concentrations 0.06–0.0006 μg/mL of the mAb pool, < 5% mean inhibition was obtained. When the inhibitory capacity of the individual mAbs 10C9, 16F1 and 3D5 was assessed in the huRBL assay, only a weak or no inhibitory capacity was observed (Figure S9). Only 16F1 was able to slightly inhibit mediator release compared to the uninhibited control

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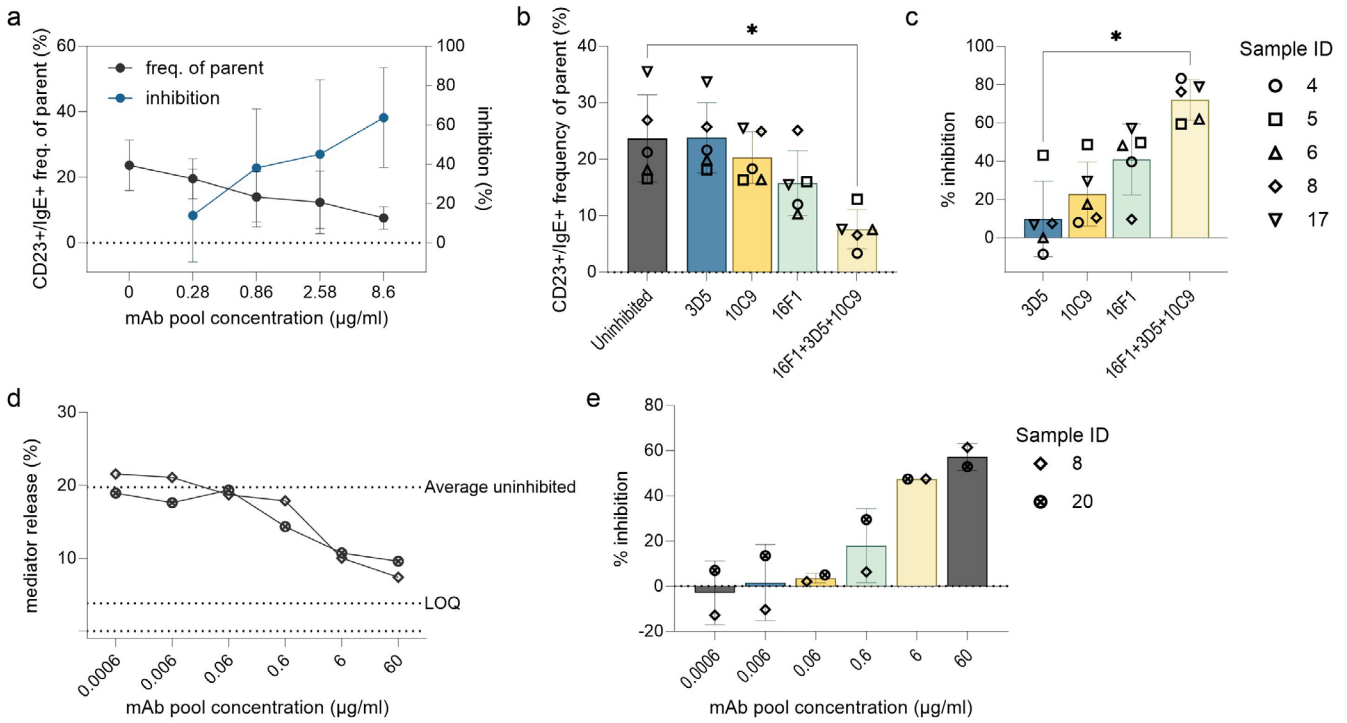

FIGURE 5 | Inhibition of IgE-binding to rFel d 7 by mAbs. A FAB assay was performed using a pool consisting of 3 mAbs (16F1, 10C9 and 3D5) that was simultaneously added with the allergen and a serum pool (a). The data are expressed as a percentage of CD23+ IgE+ EBVs (left Y-axis) and inhibition relative to the non-inhibited control (right Y-axis). The mAb pool was titrated starting at 8.6 µg/mL of each antibody. Following the initial titration, the FAB assay was repeated with the mAb pool or the corresponding individual mAbs at a concentration of 8.6 µg/mL (10-fold molar excess to rFel d 7) using individual serum samples of cat-allergic patients, $n = 5$ (b and c). Inhibition of mediator release was investigated using the RBL assay (d and e). Two individual serum samples (ID8 and ID20) were used for sensitising the huRBL cells (final dilution 1:20) overnight. For the inhibition of the allergen, 1 µg/mL of rFel d 7 was pre-incubated for 1 h with the mAb pool, titrated starting with 60 µg/mL (corresponding to a ~10:1 mAb-to-allergen ratio), followed by a 1:10 titration. The following day the cells were stimulated with inhibited and non-inhibited rFel d 7 to trigger allergen cross-linking and mediator release. LOQ and average mediator release produced by non-inhibited cells of all serum are represented by a dashed line. For statistical analysis, a repeated measures one-way ANOVA with multiple comparisons was performed (b and c). Significance was represented with asterisks: $p < 0.05$ (*).

(17.5% vs. 25.3% mean mediator release at 60 µg/mL) with a 7.8% difference in mediator release and equal to a 1.45-fold decrease. The inhibition results obtained by RBL and FAB assay were consistent and showed that the analysed mAbs 10C9, 16F1 and 3D5 effectively inhibit rFel d 7 binding to IgE, especially when applied together.

4 | Discussion

The success of allergen immunotherapy heavily relies on precise diagnostics and quality of reagents used. However, a major challenge in allergy diagnostics and immunotherapy is the lack of standardisation of commonly used allergen extracts, which are yet to be replaced by allergen components. Implementing Fel d 7 into allergy diagnostic tests adds a substantial value to the sensitivity profile, but it remains crucial to assess whether Fel d 7 is present in allergen extracts to ensure their effectiveness. This is particularly important for extracts used for allergen immunotherapy because symptoms caused by Fel d 7 would remain if Fel d 7 is not present in sufficient amounts, giving the patient the impression that AIT was not successful. Therefore, herein we have produced rFel d 7 and specific mAbs for the precise quantification of Fel d 7 content among allergen extracts.

To ensure the identity and quality of rFel d 7 that was produced in this study, we performed an in-depth physicochemical characterisation, and the MS analysis revealed a 100% sequence coverage of rFel d 7 with the theoretical sequence. The CD spectrum and FTIR spectra showed the β-sheet-dominated secondary structure and high resemblance with the previously reported recombinant Fel d 7 that was also synthesised in *E. coli*, and its crystal structure was deposited to PDB (8EPV) [38]. This verifies the identity of the herein used rFel d 7 and demonstrates the consistency and reproducibility of Fel d 7 biosynthesis in *E. coli*.

Fel d 7 is a lipocalin and it is a protein superfamily that undergoes various post-translational modifications, such as glycosylation, phosphorylation, ubiquitination and others, which affect its stability, function and cellular interactions. These post-translational modifications can regulate lipocalin activity, localization, degradation and interactions, thereby influencing processes like lipid metabolism, protein trafficking and signal transduction [39]. We acknowledge that rFel d 7 synthesised in *E. coli* lacks eukaryotic post-translational modifications, particularly glycosylation. Therefore, to assess the potential of rFel d 7 to be used for diagnostic purposes or as a reference standard in mAbs-based immunoassays, we have investigated the recognition of patients' IgE to purified rFel d 7 by ELISA, WB, and

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huRBL assay. Results of indirect ELISA showed that 73.91% of the 23 tested sera of cat-allergic patients recognised rFel d 7. This sensitisation rate is higher than other what has been reported previously [14–19], emphasising the significance of Fel d 7. The huRBL assay confirmed the biological relevance of the results by inducing mediator release upon binding of rFel d 7 to patients' IgE. However, since the assay requires high specific IgE titers for optimal performance and output, mediator release was only observed for 33.33% of the analysed samples. Nevertheless, these findings confirm the antigenic similarity of rFel d 7 compared to the native allergen.

We have developed a collection of Fel d 7-specific murine mAbs and applied them to analyse six commercial cat allergen extracts from five different manufacturers and various sources, including dander, epithelium and hair. The lowest concentrations of Fel d 7 were detected in two cat epithelium extracts from MNF#4 and MNF#5. In contrast, a cat epithelium extract from MNF#2 exhibited a significantly higher Fel d 7 concentration of 3.09 μg/mg (0.31% of total protein content), over 100 times greater than the lowest values in other extracts. The highest concentration of Fel d 7 (22.59 μg/mg, 2.26% of total protein content) was observed in the cat hair extract from MNF#5. In contrast, another cat hair extract contained only 1.19 μg/mg (0.12% of total protein content) of Fel d 7. These findings demonstrate the vast difference in Fel d 7 content in different allergen extracts. Such variations were observed in cat allergen extracts produced by different manufacturers and prepared from different sources. In comparison with a previous study [18], where the presence of Fel d 7 in commercial cat allergen extracts was analysed using polyclonal mouse anti-rFel d 7 antibodies by WB, this allergen was only weakly detected in commercial cat hair and pelt extracts, but it was readily detected in the cat saliva sample. Moreover, the authors analysed the extracts by DELFIA IgE quantitation assay (IgE inhibition assay), and the inhibition was readily detectable with the cat saliva sample, with Fel d 7 calculated to represent 0.21% to 0.37% of the total protein content.

The decision of which cat allergen extract to use for allergy diagnostics or immunotherapy might greatly impact the outcome of the results. Even though the modern allergy diagnostics use allergen components, allergen immunotherapy is still based on allergen extracts; therefore, the precise and universal standardisation of extracts is crucial for a safe treatment with a promising outcome. Currently, standardisation efforts are more focused on quantifying the allergen components rather than analysing their biological potency by assessing their ability to elicit an immune response. Herein, we demonstrate that the developed mAb-based sandwich ELISA could be an effective tool for the precise quantification of the less investigated but important allergen Fel d 7 and, thus, can be easily employed for the standardisation of cat allergen extracts. To establish the developed sandwich ELISA as a reference immunoassay, further experiments are required, including the analysis of a larger panel of cat allergen extracts, evaluation of mAb reactivity with all natural isoforms of Fel d 7, and assessment of result consistency when analysing dilution series of allergen extracts.

We applied Fel d 7-specific mAbs for the inhibition of functional binding of rFel d 7 to IgE from serum samples of cat-allergic patients by FAB and RBL assays. The results of these assays demonstrated that a significant reduction in IgE-rFel d 7 binding was achieved only using a combination of mAbs (10C9, 16F1 and 3D5) compared to individual mAbs. Since these three mAb clones recognise different epitopes of rFel d 7, the findings show the importance of steric hindrance at multiple antigen sites rather than a single location, even when increasing the mAb-to-allergen ratio. Furthermore, mAb 16F1 (IgG2a isotype) showed slightly better inhibitory effects compared to individual mAbs 10C9 or 3D5 (both IgG1) in both assays, although these differences were not statistically significant. Moreover, we suggest that mAb-based FAB and RBL assays could be employed as potency assays for the analysis and standardisation of allergen extracts. However, further experiments are needed, including the application of Fel d 7-specific mAbs for the inhibition of functional binding of IgE to natural Fel d 7 in allergen extract.

Besides analytical application, inhibition capabilities of mAbs are gaining great importance regarding recent development [40] and application [41, 42] of therapeutic anti-Fel d 1 antibodies in humans for the reduction of cat allergy symptoms. Other approaches for reducing allergy symptoms, such as immunisation of cats with recombinant Fel d 1 and universal T-cell epitope to induce neutralising antibodies [43, 44] or integrating anti-Fel d 1 polyclonal IgY from chicken eggs into their diet [45] have been reported. Overall, the approach to inhibit IgE-allergen interaction provides a new strategy for the development of advanced therapeutic applications, and our studies on Fel d 7-specific mAbs may serve as a basis for the development of blocking IgG4 antibodies for therapeutic use in the future.

Altogether, our results emphasise the need for standardisation and comprehensive analysis of allergen content and potency in allergen extracts used for skin prick tests as well as for allergen immunotherapy. We highly recommend integrating the analysis of Fel d 7 in quality control of cat hair extracts in the future.

Author Contributions

Vytautas Rudokas: performed experiments, collected, analysed, and interpreted the data, wrote and edited the manuscript, approved the final version of the manuscript. **Glorismer Pena-Amelunxen:** performed experiments, collected, analysed, and interpreted the data, edited, and approved the final version of the manuscript. **Peter Briza:** performed experiments, collected, analysed, and interpreted the data, approved the final version of the manuscript. **Lorenz Aglas:** performed experiments, collected, analysed, and interpreted the data, supervised and managed the study, edited, and approved the final version of the manuscript. **Aurelija Zvirbliene:** conceptualised, supervised and managed the study, edited and approved the final version of the manuscript.

Acknowledgements

The research was funded by the Research Council of Lithuania, contract No D-20-0929 and supported by the Research Collaboration Agreement between the University of Salzburg and Amsterdam Medical Center. L.A. received support from the Austrian Science Fund (FWF) [grant DOI: 10.55776/I5312 and 10.55776/P34207].

Conflicts of Interest

The authors declare no conflicts of interest.

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Data Availability Statement

The datasets generated and analysed during the current study are available upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Characteristics of serum samples of cat allergic patients: cat dander-specific IgE levels (in kU/L and corresponding CAP class) provided by the supplier (max value was 100 kU/L). In columns 4–7, serum samples tested with rFel d 7 in different assays are indicated Serum samples tested by WB, ELISA, or rat basophil-based assay or IgE-facilitated allergen binding assay are indicated by ✓ or left blank. The results of WB and ELISA are indicated by reactivity levels: − (no reactivity), + (low to mild reactivity), or ++ (high reactivity). **Figure S1:** DNA sequence encoding Fel d 7 that was used for the biosynthesis of rFel d 7 in *E.coli*. **Figure S2:** Flow cytometric gating strategy implemented for analysis of FAB assay results. Images are labelled 1–5 according to the chronological sequence that each gate was applied. Gate borders were applied according to the FMO controls, represented here (b). **Figure S3:** Peptide coverage of tryptic digests of rFel d 7 as determined by MS. The theoretical amino acid sequence of Fel d 7.0101 was used as reference. **Figure S4:** SDS-PAGE (a) and WB (b) analysis of rFel d 7 (2 μg) to evaluate its reactivity with IgE from serum samples of cat-allergic patients. ID numbers of the samples (as in the Supplementary Table S1) are indicated below the membrane images. **Figure S5:** Individual curves of huRBL assay for the activation of sensitised huRBL cells with rFel d 7. HuRBL cells were sensitised with 9 different cat-allergic patient serum samples diluted 1:20 in huRBL cell medium and stimulated the following day with a dilution series of rFel d 7, starting at 1 μg/mL, followed by a 1:10 dilution series. Black curves represent serum samples giving a positive response exceeding the LOQ (responders), and blue curves indicate serum samples that do not show a positive response (non-responders) (a). Curves of responsive serum samples shown in different colours per serum (b). LOQ is represented with a dashed line. **Figure S6:** WB analysis of the reactivity of mAbs 13A3 and 5C3 (0.3 μg/mL) with either rFel d 7, MBP (negative control) or *E. coli* BL21 (DE3) lysate (negative control). The images of SDS PAGE (a) and WBF (b). **Figure S7:** Analysis of the cross-reactivity of Fel-7 specific mAbs 13A3 and 5C3 with other cat and dog allergens by indirect ELISA. Recombinant MBP and rFel d 7 were used as a negative and positive control, respectively. **Figure S8:** The results of competitive ELISA. The competition for the recognition of Fel d 7 epitopes between each mAb pair is expressed as percentages, calculated from OD450 values obtained with a positive control (no mAbs) and a negative control (no rFel d 7). Values in the range of $-\infty$ to 30% indicate no competition (red), 30% to 70% indicate partial competition (yellow), and 70% to $+\infty$ indicate a full competition (green). **Figure S9:** HuRBL assay using individual mAbs (60 μg/mL) to inhibit IgE-antigen binding and mediator release. LOQ is represented with a dashed line. Error bars represent the median and range values.

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