

Atopic Dermatitis Patients with Pet Dander Sensitization Mount IgE and T cell Responses to Mammalian Cystatins Including the Human Self-Protein

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ABSTRACT

Background: Immediate as well as delayed-type hypersensitivity immune reactions to pet-borne allergens are commonly observed in atopic diseases. Further on in atopic dermatitis (AD), cross-reactivity to self-proteins is discussed to contribute to the disease. Human cystatin A and the cat allergen Fel d 3 belong to the cystatin family, an evolutionary conserved protein family. The objective of the present study was to assess cross-reactivity between mammalian cystatins and to analyze T cell responses to cystatin in AD patients sensitized to pet dander.

Methods: cDNA coding for dog cystatin was cloned from dog skin. Sera of 245 patients with IgE-sensitization to cat and dog dander were tested for IgE-binding to recombinantly expressed feline, canine, and human cystatin, respectively. Of these, 141 were also diagnosed for AD.

Results: Cystatin-specific IgE was detected in 14.7 % (36) of patients, of which 19 suffered from AD. Within the AD patients, 9 carried measurable IgE against all three cystatins. Cystatin-sensitized AD patients did not differ from non-cystatin sensitized patients in terms of disease severity, age or total IgE levels. T cell cytokine measurements showed elevated IL-4 levels after stimulation with feline and human cystatin.

Conclusion: The humoral response suggests that next to Fel d3 also the homologous protein from dog might play a role in allergy. Further on, the human cystatin appears to be capable of driving a type2 immune response in sensitized AD patients and may therefore be considered a so-called autoallergen, as it has been proposed for other evolutionary conserved proteins.

Key words: Allergy. Sensitization. Atopic dermatitis. Pet. IgE. T cell. Cross-reactivity. Autoallergy. Autoreactivity. Fel d 3. Can f 8. Cystatin. Cytokine.

RESUMEN

Antecedentes: Las reacciones de hipersensibilidad de tipo inmediato y retardado a los alérgenos que están en las mascotas son comunes en las enfermedades atópicas. En este estudio, en pacientes con dermatitis atópica (DA), se analiza la reactividad cruzada con las autoproteínas y su contribución a la enfermedad. Tanto la cistatina A humana como el alérgeno felino Fel d 3 pertenecen a la familia de las cistatinas, una familia de proteínas conservadas evolutivamente. El objetivo del presente estudio fue evaluar la reactividad cruzada entre las cistatinas de mamíferos y analizar las respuestas de las células T a la cistatina en pacientes con DA sensibilizados a la caspa de las mascotas.

Métodos: el ADNc que codifica la cistatina de perro se clonó a partir de piel de perro. Se analizaron sueros de 245 pacientes con sensibilización por IgE a la caspa de gato y perro para determinar la unión de IgE a cistatina felina, canina y humana expresada de forma recombinante, respectivamente. De estos 245 pacientes, 141 fueron diagnosticados de DA.

Resultados: Se detectó IgE específica frente a cistatina en el 14,7% (36) de los pacientes, de los cuales 19 padecían DA. Dentro de los pacientes con DA, 9 tenían IgE medible contra las tres cistatinas. Los pacientes con DA sensibilizados frente a cistatina no difirieron de los pacientes no sensibilizados con cistatina en términos de gravedad de la enfermedad, edad o niveles totales de IgE. El análisis de citocinas de células T reveló niveles elevados de IL-4 después de la estimulación con cistatina felina y humana.

Conclusión: La respuesta humoral sugiere que, además de Fel d 3, la proteína homóloga de perro también podría desempeñar un papel en la alergia. Además, la cistatina humana parece ser capaz de promover una respuesta inmune de tipo 2 en pacientes con DA sensibilizados y, por lo tanto, puede considerarse un autoalérgeno, como se ha propuesto para otras proteínas conservadas evolutivamente.

Palabras clave: Alergia. Sensibilización. Dermatitis atópica. Mascota. IgE. Célula T. Reactividad cruzada. Autoalergia. Autorreactividad. Fel d 3. Can f 8. Cistatina. Citocina.

Introduction

Hypersensitivity reactions to cat allergens are common in atopic individuals. In the general adult population, sensitization rates against cat and dog epithelia range from 10 to 12% [1, 2]. Atopic dermatitis (AD) is often observed to precede other atopic diseases like allergic asthma or rhinitis, and sensitization via the skin is therefore believed to be at the origin of the so-called atopic march. Consistently, it has been reported that 19-25 % of children affected with AD are sensitized to cat dander [3, 4]. Presently, 8 cat allergens are known, with Fel d 1 being the major cat allergen [5]. Recent epidemiological studies have shown that specific IgE to animal allergen components can be linked to symptoms. Specific IgE to Fel d 1 and Fel d 4 were significantly associated with asthma upon contact with cats [6]. A cross-sectional and longitudinal population-based study has shown that sensitization to Fel d 1 in early childhood is a predictor of cat allergy at 16 years [7]. In cat-allergic children, elevated IgE-titers to Fel d 2 and Fel d 4 were linked to atopic dermatitis [8]. Fel d 3 is a minor allergen which is recognized by 10 % of cat allergic patient sera [9] and it has not yet been analysed in regards to a possible link with clinical symptoms. Fel d 3 is an 11kDa dander protein and belongs to the protein family of cystatins, more precisely type I cystatin or cystatin A, cloned from a cat skin library [9]. Type I cystatins function as competitive inhibitors of papain-like cysteine proteases, are highly conserved among mammals and are abundantly expressed in blood and epithelial cells including keratinocytes [10, 11].

IgE-autoreactivity has been described as a common feature in AD [12, 13] and cross-reactivity appears to be an underlying mechanism. Detailed analyzes of immune responses to such so-called autoallergens suggest a contribution to the pathology of AD [14-16]. So far, cross-reactivity between autoallergens and pet-borne allergens has not been evaluated in detail. Next to serum albumins, proteins of the cystatin family represent a strong potential to cross-react between man and pet. Human cystatin A (CSTA or Stefin A) is localized mainly intracellularly, but also detectable in sweat and in the medium of cultured keratinocytes. It has functions in skin integrity, since CSTA is one of the precursor proteins of the cornified cell envelope in keratinocytes and plays a role in epidermal development and maintenance [17, 18]. CSTA interferes with foreign enzymes [19] or virus-induced apoptosis and is able to disturb herpes simplex virus 1 (HSV1) production [20]. It has been shown that the inhibitory potential on cysteine proteases by CSTA leads to reduced immune responses to house dust mite allergens, since Der p 1 and Der f 1 are in fact cysteine proteases [21, 22]. Nevertheless, its localization in

skin and lung in combination with a high enzymatic activity may predispose cystatin A to be encountered by the immune system during an ongoing inflammation. A preexisting sensitization to Fel d 3 could further boost Th2 responses and auto-reactivity to homologous human cystatin.

Therefore this study aimed to investigate IgE and T cell responses to Fel d 3 and potential auto- and cross-reactive cystatins from dog and human in sensitized patients with and without atopic dermatitis.

Material and Methods

Patients and sera

Adult patients with hypersensitivity reactions to dog or cat but no symptoms of AD were selected from the outpatient population of the National Unit of Immunology-Allergology of the Centre Hospitalier de Luxembourg, based on a history of specific IgE to cat and/or dog dander (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden). These patients were in mean 35 years old (range 13-73) and to 46% of female gender. Of these, 26 had asthma, 16 had asthma associated with rhinitis and/or conjunctivitis, 6 had rhinitis with or without conjunctivitis, while from 56 no such detailed information was available. Patients with and without hypersensitivity reactions to dog or cat that also suffered from AD according to the criteria of Hanifin and Rajka [23] were recruited at the Department of Dermatology and Allergy at Hannover Medical School based on a history of specific IgE to cat and/or dog dander. These were in mean 35 years old (range 18-72), were to 37% of female gender and the disease severity as determined by SCORAD was 41 ± 17.5 (median \pm standard deviation). AD sera are part of the BioBank of the “Klinische Forschergruppe Autoimmunität 250”, funded by German Research Foundation (DFG). All patients gave their written informed consent. The study was conducted according to the declaration of Helsinki and approved by the National Committee for Medical Research Ethics in Luxembourg and the Ethics Committee of Hannover Medical School, respectively.

Cloning of dog, cat and human cystatin

Shaved dog skin was obtained from a 14 years old female bichon dog euthanized by a veterinarian. RNA was extracted from 1 g of tissue using the RN easy Fibrous Tissue Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). cDNA was generated using the SMART RACE cDNA amplification kit (Takara Bio USA, Mountain View, CA). Forward and reverse primers were designed from a predicted dog cystatin A sequence, XM_545130.6 (3'RACE forward primer CCT GCC ACT CCA GAA GTC CAG; 5'RACE reverse primer AAA GCC TGT GAG CTC ATC ATC CTT GC). Primers were situated within the open reading frame and yielded fragments extending into the 5' and 3' untranslated region. In a second step, new primers with restriction enzyme recognition sites Nco I and Bgl II were designed to amplify the whole coding region for cloning into the pQE60 vector (Qiagen) (forward primer: 5' AGC CAC CAT GGT GCC TGG AGG CTT AAC TGA AGC C; reverse primer 5' ACA AGA GAT CTA AAG CCT GTG AGC TCA TCA TCC TTG C).

Synthetic cDNAs based on the published sequences of Fel d 3 (AF238996; Q8WNR9) and human cystatin A (NM_005213; P01040) were ordered at Eurofins Genomics (Ebersberg, Germany) and cloned into pQE60. Codons were adapted for optimal expression in *E. coli*.

Expression of recombinant cystatin

Recombinant cat, dog and human cystatins carrying a C-terminal hexahistidine tag were expressed in *E. coli* M15 (Qiagen). Protein production was induced by 1 mM isopropyl- β -D-thiogalactopyranoside (Roth, Karlsruhe, Germany) and recombinant proteins were obtained under non-denaturing conditions and purified by immobilized metal ion affinity chromatography (HisTrap HP, GE-Healthcare, Buckinghamshire, UK) as described [24]. Eluted recombinant proteins were further purified by ion exchange chromatography (Resource Q for Fel d 3, Resource S for human cystatin and Can f 8, GE-Healthcare). Fractions containing purified cystatin were pooled and dialysed against phosphate buffered saline (PBS). Purity of the recombinant protein was assessed by SDS-PAGE and silver staining, identity was confirmed by N-terminal sequencing (PPSQ; Shimadzu, Kyoto, Japan). LPS was removed from the protein preparations by applying the Endotoxin Removal Kit (Hyglos, Bernried, Germany). LPS was quantified in the final protein batches by Limulus amoebocyte lysate test (Associates

of Cape Cod, East Falmouth, MA, USA). In all T cell assays, respective amounts of LPS were applied as controls: Can f 8, 480 pg/ml; human cystatin, 33 pg/ml; Fel d 3, 88 pg/ml.

Detection of specific IgE by ELISA and ELISA inhibition

Specific IgE to Fel d 3, Can f 8 and human cystatin were quantified by ELISA, as described [25]. Briefly, each protein was coated overnight to microtiter plates (NUNC MaxiSorp™, Roskilde, Denmark) at a concentration of 5 µg/ml in PBS. Blocking buffer was 3% bovine serum albumin (BSA) (Sigma-Aldrich, Diegem, Belgium) in PBS. Patient sera were diluted 1:3 or 1:5 in blocking buffer and incubated for 2 h at room temperature on the microtiter plate. sIgE titer to cystatins were measured by using a standard curve of serial dilutions of a patient serum with a known sIgE titer to cat serum albumin. Sera with sIgE to cystatin ≥ 0.35 kU/L were rated positive. Sera of 7 healthy individuals were used as negative controls. For inhibition experiments, sera were preincubated for 2 hours with 100 µg/ml cat, dog or human cystatin before adding to the microplate.

Extraction of proteins from animal dander

Cat and dog dander were purchased at Allergon AB (Ängelholm, Sweden). Proteins were extracted in PBS at 4°C overnight from 2 g of dander, the suspension was centrifuged for 30 min at 5000g and the supernatant used in subsequent experiments.

SDS-PAGE and immunoblot

Proteins were separated under reducing conditions using 15% SDS-PAGE and immunoblotted as described [25]. Polyclonal anti-cystatin A antibody (Santa Cruz Biotechnology, Heidelberg, Germany), raised against an N-terminal peptide of human cystatin A was used to detect cystatin in cat and dog dander. The incubation with an anti-goat IgG secondary antibody labelled with alkaline phosphatase (Sigma-Aldrich) followed by addition of nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Promega, Leiden, The Netherlands) allowed to visualize cystatin-specific bands.

T cell lines

Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation and T cell lines were generated according to an established protocol [26]. More precisely, 2×10^6 PBMC were cultured in presence or absence of 2.5 $\mu\text{g/ml}$ antigen at a density of $2 \times 10^6/\text{ml}$ in Iscove's medium (Biochrom KG, Berlin, Germany) supplemented with 4% human heat-inactivated AB serum, 2 mM glutamine, 50 mg/ml of gentamicin, 100 mg/ml penicillin and streptomycin, and nonessential amino acids. After 7 days, rhIL-2 (10 U/ml) was added to the culture and after 14 days, cells were expanded with allogeneic, irradiated PBMC (50Gy) as feeder cells in the presence of phytohemagglutinin (10 $\mu\text{g/ml}$) and rhIL-2 (10 U/ml). After four weeks, T cell lines were stimulated with concavalinA (10 $\mu\text{g/ml}$) and culture supernatants were harvested 18h after stimulation. ELISAs were performed according to the manufacturers' instructions and analyzed using a plate reader (FluoStar optima, BMGLabtech, Ortenberg, Germany). ELISA kits for the detection of IL-4, IL-17, and IL-22 were purchased at R&D (Minneapolis, MN, USA) and IFN- γ at ebioscience (San Diego, CA, USA).

Screening for genetic variants within the FLG gene

The DNA of cystatin-sensitized AD patients was analysed towards variants within the FLG gene that have been described to be associated with AD (2282del4, S3247X, R501X, and R2447X) by Sanger sequencing of PCR products. PCR Primers were applied as follows: R501X: Forw CTGACTCTGCCCATGGACG Rev CCGGGTGTCCACGAA; 2282del4 Forw TCCCGCCACCAGCTCC Rev CCCTGAACGTCGAGACCT; R2447X Forw GTCAGCAGGAAGGTCTGGAC Rev GCCTTCCTCCACTGCTTGAC; S3247X Forw GATCCAGTGTGAGCCAGGAC Rev CTGAACGTCACAGACCTTCCC.

Statistics

To calculate differences among two groups that do not follow Gaussian distribution, the Mann-Whitney test was applied (GraphPad Prism, Version 5.02 and 7.04, GraphPad, La Jolla, CA, USA). Contingency tables and Fisher's exact test were used to analyse differences in IgE prevalence to a specific cystatin among groups.

Results

IgE-sensitization to pet dander is associated with a higher SCORAD

Patients with pet dander sensitization were identified by the presence of specific IgE (sIgE) to cat and/or dog dander. PhadiaImmunoCAP reagents e1 and e5 are part of the standard aeroallergen panel (PhadiaImmunoCAP sx1) used to assess aeroallergen-sensitization. In total, 245 patients were identified with significant amounts of IgE to cat or dog dander. Atopic patients with and without AD were grouped and analyzed separately. Patients without detectable respective IgE served as negative controls in the following experiments. Interestingly, in AD patients an IgE-sensitization to dog and/or cat was associated with a higher disease severity as indicated by SCORAD (Figure 1A; $P=.0455$; SCORAD cat/dog-sensitized 44.0 ± 18.0 vs. cat/dog-non-sensitized 35.5 ± 19.5 median \pm standard deviation). This association appears to be independent from the concentration of dog cat dander IgE within the serum or within the total IgE (Figure 1B-D).

Dog cystatin is expressed in dog dander

In order to assess the possibility of the existence of an allergen homologue to the cat allergen Fel d 3 in dog dander, cystatin was searched for by means of western blotting of cat and dog dander extracts and applying a commercially available polyclonal antibody. In both cat and dog dander extracts several bands were detectable in the predicted size range of 11 kDa and higher (Figure 2A). The existence of a dog cystatin gene had been predicted upon genetic similarities, which we could use as a starting point to clone the sequence by RACE-PCR. The resulting cDNA sequence was submitted to the European Bioinformatics Institute (EMBL-EBI, Hinxton, UK) and is now accessible under LT994967 (cDNA) and F1PHB6 (protein). The allergen name Can f 8 was allocated by the WHO/IUIS Allergen Nomenclature Sub-Committee. To study cystatin-specific IgE binding and T cell cytokine secretion, Fel d 3, Can f 8 as well as human cystatin were expressed in *E. coli* (Figure 2B). Alignment of the amino acid sequences of dog, cat and human cystatin A shows large stretches of highly conserved sequences (Figure 2C). The sequence identities between Fel d 3 and Can f 8 account for 83.7%, Fel d 3 and human cystatin share 75.5% sequence identity, and dog and human cystatin 78.6%.

Patients with sensitization to animal dander are IgE-sensitized to one or several mammalian cystatins

Sera from 245 patients with significant amounts of IgE to cat or dog dander (see supplemental Figure 1) of which 141 also suffered from AD were subjected to IgE-ELISA applying the recombinant cat, dog, and human cystatins, respectively. Cystatin-specific IgE was detected in 36 patients resembling 14.7 %, of which 19 suffered from AD (Figure 3A). Within the AD patients, most patients were sensitized against more than one cystatin, and 9 carried measurable sIgE against all three cystatin species (Figure 3B). Non-AD patients were more frequently sensitized to Fel d 3 (12.5%) and Can f 8 (14.4%), and less frequently to human cystatin (5.8%), but the difference to AD patients was not significant (Figure 3). Of the AD patients, 7.8% were sensitized to Fel d 3, 10.6% to Can f 8 and 9.9% to human cystatin. Of interest, patients with exclusive IgE-sensitization to the human cystatin (without parallel sensitization to pet-borne cystatins) can be found in AD only, underlining that IgE-autoreactivity is a hallmark of AD.

IgE-sensitization to cystatin is not associated with disease severity or sIgE levels to pet dander

Regarding the clinical characteristics of pet dander-sensitized AD patients, IgE-sensitization to cystatin was not associated with titers of sIgE to cat or dog dander (Supplemental Figure 3). Also total IgE levels, age or the severity of AD, as determined by SCORAD, was not associated with measurable sIgE to cystatins (supplemental Figure 2). Regarding comorbidities, 6/19 AD cystatin-sensitized patients also suffered from asthma, while 4/19 did not (for 9/19 no data were available).

No elevated frequencies of FLG variants in AD patients with IgE-sensitization to cystatin

Four genetic variants of the FLG gene, which encodes the well-investigated skin barrier protein filaggrin, have been associated with the development of AD patients, namely 2282del4, S3247X, R501X, and R2447X [27, 28]. In epidemiological studies it was observed that having a cat at home enhances the effect of carrying the 2282del4 FLG variant on the development of IgE sensitizations [29] and AD [30]. To investigate, whether FLG variations occur with higher frequencies in cystatin-sensitized AD patients, the four gene loci were analysed by Sanger sequencing. Of 19 AD patients with detectable IgE to cystatin, four carried the heterozygous

variant 2282del4, and no patient was carrier of the FLG variants S3247X, R501X, or R2447X. Since the 2282del4 variant has been reported to occur with frequencies of 13.6 % and 16.1 % in AD patients in Germany [31] and the Netherlands [29], respectively, our data do not point towards a specific association with sensitization to cystatin.

Human, feline and canine cystatin share B cell epitopes

In order to investigate IgE cross-reactivity, serum IgE binding to recombinant cystatins was assessed by ELISA with or without pre-incubation with different cystatins. In some patients, Fel d 3 was able to inhibit IgE-binding to Can f 8 and human cystatin, pointing to a primary sensitization to cat (Figure 4 upper panel and lower panel, middle), whereas in another patient, primary sensitization seems to originate from Can f 8 (Figure 4, lower panel, right). IgE directed to human cystatin recognize mostly cross-reactive epitopes as in only one of the patient sera, human cystatin is able to substantially inhibit IgE binding to dog or cat cystatin (Figure 4, lower panel, middle). As this patient displayed the highest amount of cystatin-specific IgE within the group of AD patients, this raises the question on the impact of this reactivity on his disease.

T cell reactivity to cystatin in sensitized patients

To investigate the quality of the adaptive immune response to human cystatin, PBMCs were stimulated by recombinant cystatins over 4 weeks to propagate putative specific T cells. Since T cell autoreactivity has been described to occur frequently in AD, the T-cellular response was investigated in atopic patients suffering from AD and respective IgE-sensitization. Parallel *in vitro* stimulation with LPS amounts resembling the detected endotoxin contamination of the respective recombinantly expressed cystatins served as control cells. Subsequently, cells were mitogen-activated and the cytokines IL-4, IL-17, IL-22, and IFN- γ were assessed by ELISA.

Cells of sensitized donors released in response to human cystatin in median 106.0 ± 85.0 pg/ml IL-4, and in response to Fel d 3 20.0 ± 184.6 pg/ml IL-4. These cytokine levels were significantly higher compared to control LPS-stimulation resulting in 7.9 ± 42.9 pg/ml IL-4. In healthy donors or AD patients without IgE-sensitization to cystatin, no cytokine secretion was measurable that

exceeded significantly the control LPS-stimulation. Due to high inter-patient variability the data are displayed as stimulation ratio (index) to enhance visibility (Figure 5).

Discussion

Cystatins are evolutionary conserved among mammals, which raises the question on their presence in animal dander other than cat and their role in IgE-cross-reactivity. In the original characterization of Fel d 3, 10% of the sera were reactive with the *E. coli* produced protein, whereas 60-90 % reacted in the plaque immunoassay where the allergen was expressed as a bacterial fusion protein[9]. In the present work, we identified a homologue of Fel d 3 in dogs and successfully cloned dog cystatin, Can f 8, from dog skin.

Within the 245 patients analyzed in this study, we detected IgE sensitizations to Can f 8 in about 12 % of pet dander-sensitized patients, whilst 10 % percent were sensitized to Fel d 3. The *in vitro* experiments performed here suggest a certain degree of IgE-cross-reactivity, since most patients' Fel d 3 specific IgE could be trapped by Can f 8 and vice versa. Our data suggest that cystatins represent - just like lipocalins or albumins- are family of pet-borne allergens.

Furthermore, we detected sIgE against human cystatin A in about 8 % of pet dander-sensitized individuals, so it can be concluded that patients are frequently mono- or poly-cystatin-sensitized. In our hands, IgE-cross-reactivity could be observed *in vitro* between all 3 cystatins. However, a prerequisite for human cystatin to act as an antigen is its presentation via MHC complexes, so the question remains whether cystatin is part of the human peptidome. Harnessing the power of high-throughput proteomics, T cell epitopes presented by MHC-molecules on the surface of antigen-presenting cells can be identified nowadays by mass spectrometry. By this, cystatin has been shown to be presented via MHC I and also II in healthy donors[32-36]. We and others discussed earlier, that during an ongoing inflammatory episode of AD, cellular components can be released into the surrounding skin tissue as a consequence of cell destruction by scratching [37]. The surrounding type-2 inflammatory milieu is believed to facilitate a sensitization subsequently, whilst inborn properties of the antigens may further foster this procedure[38, 39]. The fact that exclusive IgE-sensitization to the human cystatin without parallel sensitization to pet-borne cystatins was only detected in atopic patients with AD underlines this theory. Early exposure to cats was found to increase the odds ratio to develop atopic dermatitis during childhood in individuals carrying a mutation in the filaggrin gene.

However, in our set of patients, no association with the four most common filaggrin mutations was detectable.

T cell responses to cat allergens have been investigated in several studies, while mostly focusing on the major allergen Fel d 1. MHC-class-II-multimer staining of Fel d 1 specific T cells revealed a central memory phenotype with low expression of skin-homing markers [40], but a strong Th2 polarization as determined by expression surface markers and secretion of IL-5 [41, 42]. Specific allergen immunotherapy (SIT) is indeed considered a promising option for sensitized patients who cannot avoid the allergen, however, newer studies focus solely on Fel d 1 [43-45]. Interestingly, SIT with synthetic Fel d 1 peptide epitopes results in a reduced expression of the Th2 cell surface marker CCR4 on Fel d 1-specific T cells [46]. Matching these observations on Fel d 1, Fel d 3 as well as human cystatin led in our hands to Th2 T cell responses in sensitized individuals, namely the induction of IL-4. The lack of IL-4 in Can f 8 stimulated samples remains speculative, but may be a result of the relatively small numbers of individuals applied in this assay.

The immune responses of pet-derived lipocalins have been compared to homologous human proteins also in the past. While in cells from healthy donors human lipocalins appear to induce a Th1 response, pet allergens led to increased levels of IL-13 [47]. It has therefore been discussed that intrinsic properties of pet-derived lipocalins predispose them to induce a type 2 response. In individuals carrying IgE-sensitizations to pet lipocalins, the immune response has been described to be dominated by IL-4 and IL-5, both towards pet or human lipocalin [48].

Cross-reactivity to pet allergens appears therefore a plausible mechanism to underlie the observed sensitization to several homologous cystatins. For allergens that are grouped into allergen families according to their structure or function, cross-reactivities can frequently be observed. These can also affect different atopic diseases, such as food, skin and respiratory allergy. One of the best studied cross-reactions between pollen and plant food is the birch pollen related syndrome, harboring a significant clinical impact [49]. The clinical impact of cross-reactivity to human autoallergens is difficult to estimate. Although in the present study we were able to show IgE binding and T cell proliferation to human cystatin, disease severity was not associated with sIgE to cystatin. One may presume a clinical relevance of cross-reactivity between fungal and human allergens, since latter have been proven to evoke skin lesions in sensitized patients' skin. More precisely, recombinantly produced human manganese superoxide dismutase (MnSOD) has been applied to patient's skin in the context of an atopy

patch test (APT) [50]. The APT represents the test to assess the late type (type IV-like) hypersensitivity skin reaction to protein allergens in a controlled fashion [51]. Although the responses to single autoallergens may appear rather modest, the high frequency of sensitizations against self of 23-91% AD patients [13] and the large number of described autoallergens with a role in AD (> 140) [12] may result in a significant impact, fueling the inflammation in a chronic manner. We therefore believe that the focusing on Fel d 1 with regard to ongoing studies and treatment options may bear a certain risk, since sparing minor allergens like Fel d 3.

In conclusion, we identified cystatins as a new family of cross-reactive animal allergens. Although a majority of AD patients with sIgE to Fel d 3 and/or Can f 8 had sIgE to human cystatin, an association with disease severity could not be established.

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Conflicts of interests

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Figure legends

Figure 1

IgE sensitization to cat and/or dog dander in AD patients is associated with a higher disease severity. A) IgE sensitization data from 40 AD patients without IgE sensitization to dog and cat dander (e1 and e5 Cap class 0) was used for statistical comparison to 141 AD patients with IgE sensitization to cat and/or dog dander as detected by PhadiaImmunoCAP. B-D) Higher disease severity was not associated with the amount of IgE to cat or dog dander. In B), patients are stratified according to ThermoFisherPhadiaImmunoCAP class as depicted. SCORAD: Scoring Atopic Dermatitis. * $p < .05$, Mann-Whitney test.

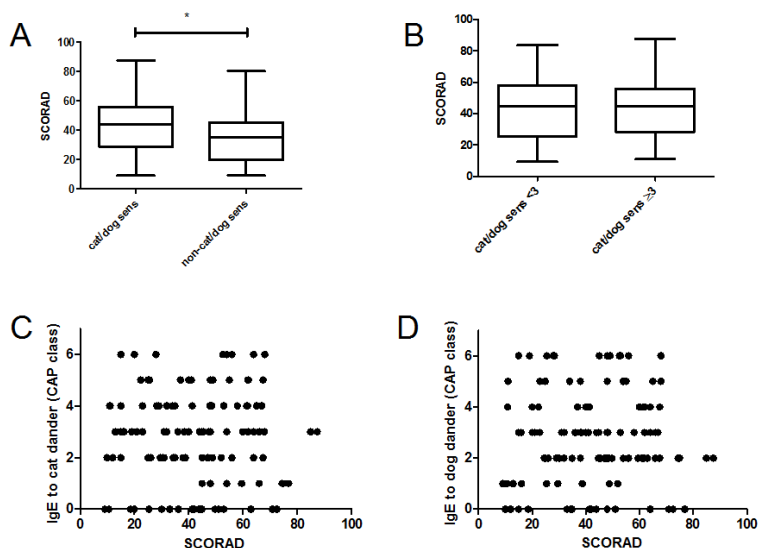


Figure 2

Dog cystatin is present in dog dander and it is highly homologous to cat and human cystatin. A) Coomassie staining (left) of cat and dog dander extracts as well as immunoblotting (middle, right) applying a commercially available polyclonal anti-cystatin antibody (Ab). N: negative control. B) Recombinantly expressed human (H), dog (D), and cat (C) cystatins are analysed by Coomassie-stained SDS-PAGE under reducing conditions. M: size marker. C) Sequence alignment of cat (Q8WNR9), dog (F1PHB6) and human cystatin (P01040). Amino acid residues shared between all 3 cystatins are shaded in grey.

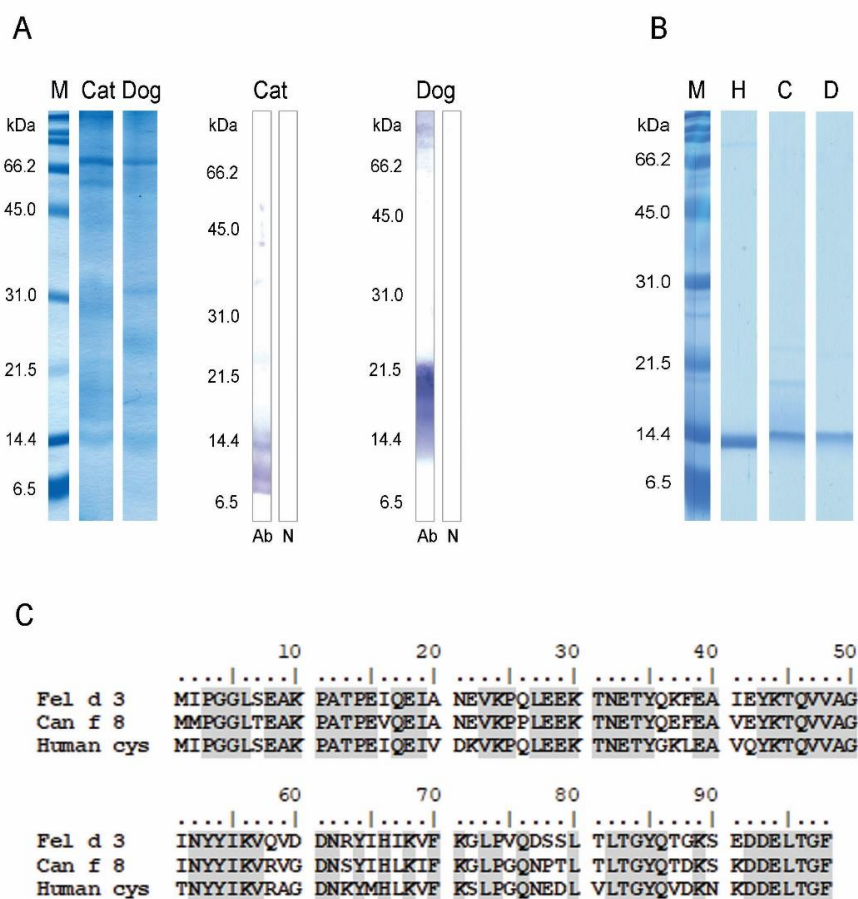


Figure 3

Detection of specific IgE to cat (Fel d 3), dog (Can f 8), and human cystatins by ELISA. A) Frequency of patients with detectable IgE-sensitization to different cystatins within cat- and/or dog dander-sensitized individuals. Patients with (n=141) or without (n=104) atopic dermatitis (AD) are depicted separately. B) Schematic drawing to visualize the degree of polysensitization of IgE-sensitized AD and non-AD patients to cystatins as indicated.

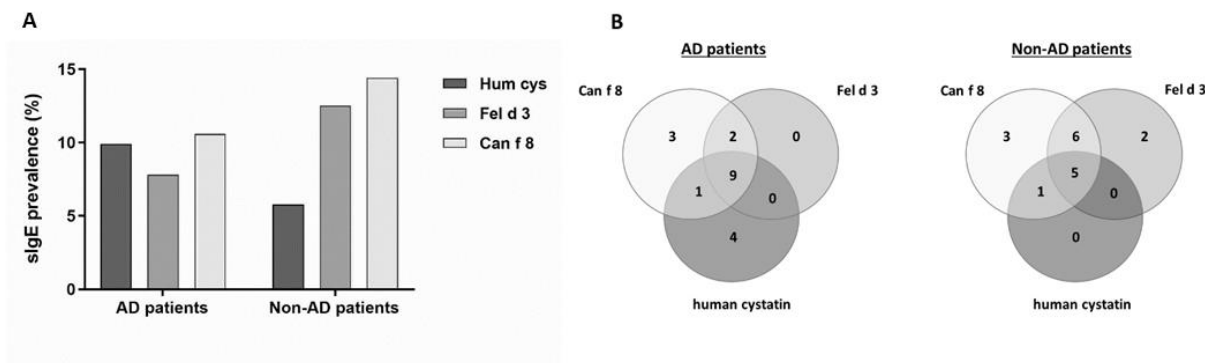


Figure 4

IgE-inhibition assays in sera of patients sensitized to cystatin. Inhibitions were done with 100 µg/ml, color code refers to coated allergens. Each graphic represents a single patient.

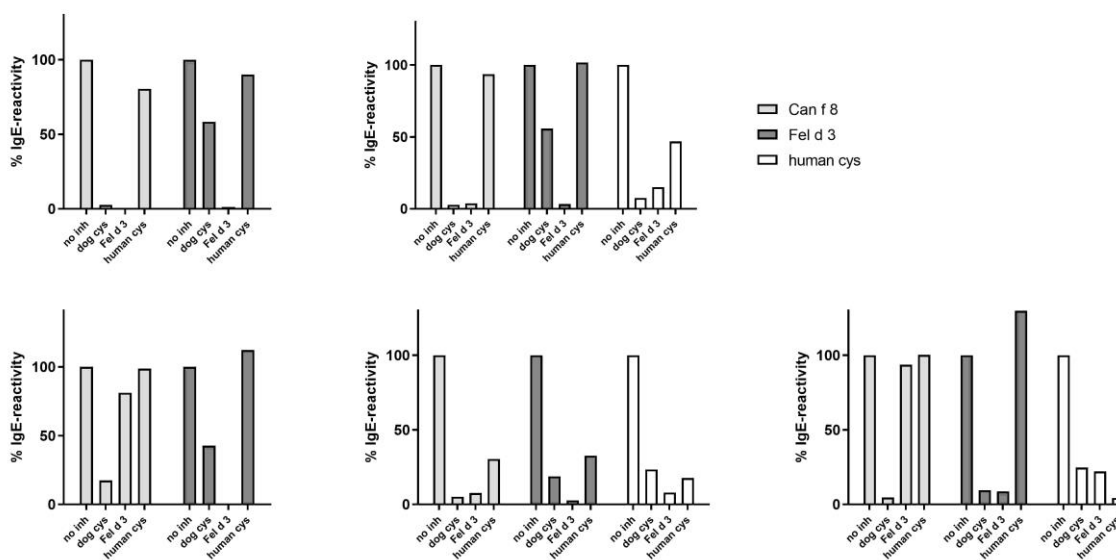


Figure 5

Cytokine profiling of 3-week cystatin-propagated T cell lines (TCL) derived from sensitized AD patients, non-sensitized AD patients, and healthy donors by means of ELISA. For means of comparison, T cells were propagated for 3 weeks by LPS amounts resembling the endotoxin contamination of the respective recombinantly expressed cystatin. Data are displayed as stimulation ratio of T cell line/non-stimulated T cells (index) to enhance visibility. n=5, *p<.05, Mann-Whitney test.

